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Hypothalamic and medullar mechanisms for long-term autonomic regulation of arterial blood pressure

Vera Lúcia Poço Geraldes

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Vera Lúcia Poço Geraldes

Supervisor: Professor Isabel Rocha
Co-supervisor: Professor Tiago Fleming Outeiro

PhD in Biomedical Sciences
Physiology

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Lisbon. The work is original, except where indicated by special reference in the text, and no part of the dissertation has been submitted for any other academic award.

Any views expressed in the dissertation are those of the author.

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In memoriam of my father,

Álvaro Geraldes (1944-2013)

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A note to the readers,

This thesis consists of several chapters and other complementary sections. Chapters 1 and 2 contain an organized review of the literature that will introduce the reader to the field and lead him/her naturally to the objectives, working hypotheses, methodologies of work, results and their specific discussion, which are shown on chapter 3. A final Discussion on Chapter 4 establishes the novelty and importance of our contribution to this field of knowledge, considering alternate interpretations of the data and study limitations. On Chapter 5, future lines for experimentation are referred.

The present thesis has two appendices: Appendix I consist of information on autonomic function and organization that is relevant to the thesis but is not necessary to understanding the text whereas in Appendix II the original print of the papers is presented. The References are collected at the end of the thesis. Complementing this organization and presented in the first pages are the dedication, acknowledgments, authorship, indices of figures and tables, list of abbreviations and the summary of the thesis, both in English and Portuguese.

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AUTHORSHIP

The results presented in this dissertation are or will be supporting the following manuscripts:

1. Geraldes V, Gonçalves-Rosa N, Liu B, Paton JF, Rocha I. Chronic depression of hypothalamic paraventricular neuronal activity produces sustained hypotension in hypertensive rats. Exp Physiol. 2014 Jan; 99(1):89-100.

2. Geraldes V, Gonçalves-Rosa N, Liu B, Paton JF, Rocha I. Essential role of rostral ventrolateral medullary neuronal activity in the long term maintenance of hypertension in conscious spontaneously hypertensive rats. Under review in: Autonomic Neuroscience: basic & clinical.

3. Geraldes V, Gonçalves-Rosa N, Laires R, Paton JF, Outeiro TF, Rocha I. Evaluation of gene expression changes in major target organs induced by chronic depression of central sympatho-excitatory regions in hypertensive rats. Submitted to Acta Physiologica.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ADH	Antidiuretic hormone
AGRP	Agouti related protein
AHT	Arterial Hypertension
Ang	Angiotensin
ANP	Arterial natriuretic peptide
AP	Arterial pressure
Arc	Arcuate nucleus
AT-1	Angiotensin II type 1 receptors
AT-2	Angiotensin II type 2 receptors
AV3V	Anteroventrolateral region of 3rd ventricle
BP	Blood pressure
bpm	Beats per minute
BSA	Bovine serum albumin
CNS	Central nervous system
CPA	Cervical Pressure Area
CRF	Corticotrophin releasing factor
CVLM	Caudal ventrolateral medulla
DBP	Diastolic blood pressure
DMNV	Dorsal motor nucleus of the vagus
DOCA	Deoxycorticosterone
DA	Dopamine
EDTA	Ethylenediaminetetracetic acid
eGFP	Enhanced green fluorescent protein
EHT	Essential Arterial Hypertension
ECM	Extra Cellular Matrix
ESH	European Society of Hypertension
ESC	European Society of Cardiology

FFT	Fast Fourier transform
GABA	γ-aminobutyric acid
GLU	Glutamate
HF	High frequency
hKir2.1	Human Kir2.1
HR	Heart rate
I.P.	Intraperitoneal
I.V	Intravenous
IL	Interleukin
IL-6	Interleukin-6
IML	Intermediolateral
iNOS	Inducible nitric oxide synthase
LF	Low frequency
LF/HF	Low frequency/High frequency ratio
LPBN	Lateral parabrachial nucleus
LPS	Lipopolysaccharides
LVV	Lentiviral vector
LVV-eGFP	LV-Syn-Eff-GAL4BS-Syn-Tetoff; LV-TREtight-Egfp
LVV-hKir2.1	LV-Syn-Eff-GAL4BS-Syn-Tetoff, LV-TREtight-hK_{ir}2.1-IRES-eGFP
MBP	Mean blood pressure
MCPA	Medullo-cervical pressor area
mmHg	Millimetres of mercury
mRNA	messenger RNA
ms	Millisecond
NA	Nucleus ambiguous
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
NTS	Nucleus tractus solitari

OT	Oxytocin
ORX	Orexin
PAG	Periaqueductal grey
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNS	Parasympathetic nervous system
PVN	Paraventricular nucleus of the hypothalamus
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
RQ	Relative quantification
RSNA	Renal sympathetic nerve activity
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
RVLM	Rostroventrolateral medulla
S.E.M	Standard error of the mean
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rat
SHRSP	Stroke prone spontaneously hypertensive rat
SNA	Sympathetic nerve activity
SNS	Sympathetic nervous system
Sp Cord	Thoraco-lumbar spinal cord
SPNs	Sympathetic preganglionic neurons
SYN	Synapsine
TBS-T	Tris-buffered saline
TPR	Total peripheral resistance
VP	Vasopressin
WKY	Wistar Kyoto rat

RESUMO

A etiologia da hipertensão essencial é multifactorial e não está completamente esclarecida; no entanto, aparentemente, a persistência de uma actividade simpática elevada é um dos principais contributos para o aparecimento, desenvolvimento e manutenção da hipertensão arterial essencial (HTA) de origem neurogénica.

De facto, a partir de dados, obtidos por aplicação de técnicas de microneurografia e de *spillover* de noradrenalina a modelos animais de hipertensão e a doentes hipertensos, observa-se que a influência simpática sobre o sistema cardiovascular está muitas vezes aumentada quando a pressão arterial está persistentemente elevada.

No entanto, os mecanismos precisos responsáveis pela activação simpática na hipertensão essencial ainda precisam ser esclarecidos, uma vez que estes são complexos e multifactoriais. Podem, no entanto, ser discutidas várias possibilidades, algumas delas destacando o papel de substâncias libertadas para a circulação e outras dando ênfase aos mecanismos com origem no sistema nervoso central.

Factores humorais como adipocitocinas, concentração de O_2 e de CO_2 no sangue, factores endoteliais e aldosterona também têm sido implicados na simpatoexcitação mas o mais estudado deles é a angiotensina II, devido à sua posição na cascata de acontecimentos pela qual o rim regula a pressão sanguínea.

Assim, uma das hipóteses é que a activação do sistema nervoso simpático depende da concentração circulante de angiotensina II uma vez que a angiotensina exerce efeitos excitatórios centrais, facilita a libertação de noradrenalina e amplifica a resposta adrenorreceptora a estímulos não apenas nos indivíduos com elevados níveis de renina e angiotensina, mas, também, em indivíduos com baixos níveis de renina.

Outra possibilidade é o facto da hiperactividade simpática ser devida à resistência à insulina, uma vez que é frequente a presença simultânea de hipertensão com hiperinsulinémia que se sabe aumentar o tráfego simpático e a libertação de noradrenalina. No entanto, o recíproco também é verdadeiro, pelo que será difícil determinar se é a simpatoexcitação que precede a resistência à insulina ou o contrário.

Uma terceira possibilidade poderá ser o facto de a activação simpática estar relacionada com disfunção baroreflexa, uma vez que a hipertensão arterial se caracteriza por um *resetting* da modulação baroreflexa da pressão arterial e do tráfego simpático no sentido de valores tensionais elevados, um mecanismo que funciona mais para manter do que para reduzir o aumento da pressão arterial uma vez que, aparentemente, também a acção de outros arcos reflexos cardiovasculares que interferem no fluxo simpático para os vasos, na libertação de renina e de noradrenalina estão inibidos.

Uma quarta possibilidade deve-se à manutenção de um fluxo simpático aumentado, de origem central, que poderá estar relacionado com excessivo controlo subcortical decorrente do stress ambiental persistente.

A presente tese baseia-se no facto de que o aumento da actividade simpática pode resultar de uma actividade inapropriada mais elevada do tónus simpático em centros cerebrais. Dois deles são particularmente importantes na regulação cardiovascular na hipertensão: a face rostroventrolateral do bulbo (FRVLB) e o núcleo paraventricular do hipotálamo (PVN).

A primeira é o maior grupo de neurónios do tronco cerebral com actividade tónica espontânea que controlam a actividade do sistema nervoso simpático. Através de projecções directas para a medula espinhal, os neurónios da FRVLB exercem um efeito estimulatório tónico contínuo para os neurónios motores pré-ganglionares da medula espinhal que regulam directamente a actividade do sistema nervoso simpático (SNS). Na hipertensão neurogénica, os neurónios da FRVLB exibem uma frequência de disparo anormalmente aumentada, o que leva ao aumento da actividade do SNS, que promove a vasoconstrição e o aumento da pressão arterial. A FRVLB também desempenha um papel fundamental na modulação do baroreflexo em condições fisiológicas e patológicas. Além disso, a actividade neuronal da FRVLB é extrinsecamente controlada por outras áreas cardiovasculares no sistema nervoso central, uma das quais é o PVN. O PVN surge como um dos principais reguladores deste *output* para o sistema nervoso autónomo e endócrino.

O PVN apresenta projecções recíprocas para a FRVLB e projecções directas para o núcleo intermediolateral simpático da medula espinhal, daí que a estimulação dos neurónios do

PVN aumenta a actividade da FRVLB e a pressão arterial. Muitos neurónios do PVN que se projectam para a FRVLB também exibem uma autorritmicidade intrínseca, e a frequência de disparo destes neurónios está intimamente relacionada com a frequência de descarga simpática.

As extensas projecções do PVN a regiões centrais (FRVLB, área postrema, NTS e núcleo intermediolateral da medula espinhal) indicam que o PVN desempenha um papel importante na modulação da actividade da FRVLB e do fluxo simpático. O PVN recebe informação de várias regiões do sistema nervoso central, incluindo aqueles associados com o controlo osmótico, apetite, metabolismo energético e stress, bem como de outras áreas que exercem efeitos sobre a pressão arterial. Assim, é evidente que o papel do PVN é integrar a informação a partir de diversas origens e modificar a actividade da FRVLB de acordo com a informação recebida.

Igualmente, foi demonstrado que a lesão electrolítica do PVN em ratos espontâneamente hipertensos (SHR) induz uma redução aguda da actividade simpática, juntamente com uma diminuição da pressão arterial. Outros estudos de fase aguda, realizados sob anestesia geral, mostraram que injecções de muscimol no PVN diminuem a pressão arterial e a actividade nervosa simpática renal, tanto em SHR como em ratos Wistar, indicando que esta área é tonicamente activa no controlo da pressão arterial e da actividade simpática periférica tanto na hipertensão como na normotensão. Além disso, a relação entre os neurónios do PVN e da FRVLB para o controlo simpático sugere que a descarga espontânea pode ser modificada por alterações na frequência intrínseca de despolarização ou através de modificações do balanço de excitação e inibição de informação nervosa aferente.

Assim, com o presente trabalho pretendeu-se modular a actividade simpática em áreas centrais - PVN e FRVLB - e estabelecer o papel desta modulação no tônus simpático, pressão arterial, reflexos cardiovasculares e nas alterações de sinalização nos órgãos alvo-hipertensivos.

Para isso, provocou-se a diminuição crónica da excitabilidade celular nestas duas áreas centrais num modelo animal de hipertensão através da sobre-expressão de canais de potássio induzida por um lentivírus monitorizando-se os valores tensionais, o output

autonómico, as funções baro e quimiorreceptora e a sinalização molecular nos órgãos-alvo da hipertensão. Como área controlo utilizou-se a área pressora bulbo-cervical (APBC) localizada na região ventrolateral da junção bulbo-cervical e cujos neurónios projectam para os neurónios simpáticos pré-ganglionares. Esta área que tem pouca acção sobre o sistema cardiovascular e que não tem qualquer transmissão neuronal para a FRVLB ou para regiões suprabulbares foi recentemente descrita como uma área simpatoexcitatória com grande interferência na função respiratória.

Os resultados mostram que a sobre-expressão crónica de canais de potássio no PVN e na FRVLB em ratos SHR conscientes causou uma diminuição acentuada e sustentada da pressão arterial e do output simpático avaliado indirectamente pela diminuição da potência da banda das baixas frequências (LF) de pressão arterial sistólica (PAS). No PVN, em particular, observou-se uma remodelação reversa das funções baro e quimiorreceptora que se aproximaram da função fisiológica normal. Curiosamente, não se observaram modificações nestas funções com a intervenção na FRVLB, onde estes reflexos integram primariamente.

Ocorreram igualmente alterações de sinalização nos órgãos-alvo da hipertensão, coração, rim e vasos. De facto, a manipulação central que promoveu a diminuição dos valores de pressão arterial e da actividade simpática, também, afectou a expressão génica nos órgãos-alvo, principalmente através do aumento da expressão dos genes angiotensinogénio e receptores da angiotensina II tipo 2 (AT-2) no rim e da diminuição de expressão de receptores da angiotensina II tipo 1 (AT-1) no coração.

Estes resultados destacam o PVN e a FRVLB como locais importantes para o controlo da pressão arterial na hipertensão neurogénica, e espera-se que ao identificar a sua função específica nesta patologia elas se possam constituir alvos realistas para intervenções terapêuticas mais dirigidas na hipertensão.

Em conclusão, o presente estudo mostra que a intervenção na excitabilidade neuronal de áreas centrais simpatoexcitatórias através da manipulação genética da expressão de canais de potássio é capaz de alterar a pressão arterial periférica a longo prazo. Isto ocorre pela remodelação do fluxo simpático e por alterações de sinalização que ocorreram nos órgãos-alvo para manter a homeostase cardiovascular. Os nossos dados,

obtidos a partir de um modelo animal, dão perspectivas sobre os mecanismos fisiopatológicos envolvidos na etiologia da hipertensão arterial neurogénica e poderão proporcionar novas intervenções terapêuticas a nível central do sistema nervoso autónomo para controlo da simpato-excitação e dos danos funcionais nos orgãos periféricos.

Palavras-chave: Hipertensão Arterial, Sistema Nervoso Simpático, órgãos-alvo, núcleo paraventricular do hipotálamo (PVN), face rostroventrolateral do bulbo (FRVLB), Lentivirus (LVV), barorreflexo e quimioreflexo.

xxx

ABSTRACT

The aetiology of essential hypertension is multi-factorial and not completely understood. Apparently, the persistence of elevated sympathetic activity is one of the major contributors to the onset, development and maintenance of neurogenic arterial hypertension (AHT).

From experimental models of hypertension and hypertensive patients data using microneurography and norepinephrine spillover techniques, there is evidence that the sympathetic influence upon the cardiovascular system is often increased when blood pressure is chronically elevated.

The mechanisms responsible for the sympathetic activation in essential hypertension are complex and multifactorial and remained to be completely elucidated. However, several working hypothesis can be discussed, some of them stressing the role of humoral substances and others concentrated on brain mechanisms.

Humoral factors like adipokines, O2 and CO2 blood concentration, endothelial factors and aldosterone have also been implicated in sympathoexcitation but the most studied of them is angiotensin II due to its position on the cascade of events by which the kidney regulates blood pressure.

Thus, one hypothesis is that the sympathetic nervous system activation depends on the circulating angiotensin II concentration, since it exerts central sympathoexcitatory effects, promotes the release of norepinephrine and amplifies the adrenoreceptor response to stimuli not only in subjects with elevated levels of renin and angiotensin, but also in subjects with low levels of renin.

Other hypothesis is that the sympathetic hyperactivity may be due to insulin resistance, since the presence of hypertension is often associated with hyperinsulinemia and is known that insulin resistance/hyperinsulinemia increases the sympathetic traffic and the release of norepinephrine. However, the reciprocal is also true, so it is difficult to determine, in this case, whether it is the sympathoexcitation that precedes insulin resistance or otherwise.

A third assumption links sympathetic activation with baroreceptor reflex function. In fact, sympathetic activation is associated with baroreflex impairment, since hypertension is characterized by baroreflex remodeling and sympathetic nerves traffic resetting towards high blood pressure values. The major objective of this physiological adaptation is not to reduce the increased blood pressure values but to maintain a new steady-state of high values since, apparently, also the action of cardiac reflex arcs that may affect the sympathetic outflow to the vessels, the release of norepinephrine and renin is inhibited.

A fourth theory deals with the maintenance of an increased central sympathetic outflow due to excessive subcortical control caused by persistent excessive environmental stress.

This thesis is based on the possibility that increased sympathetic activity observed in hypertension may also result from an inappropriately elevated sympathetic drive from brain centres. Two of them are particularly important to cardiovascular regulation in hypertension: the rostroventrolateral medulla (RVLM) and the paraventricular nucleus of the hypothalamus (PVN).

The first is the major brainstem cluster of neurons with spontaneous tonic activity that controls peripheral sympathetic activity. Through direct projections to the spinal cord, RVLM neurons provide tonic drive to the spinal cord preganglionic motor neurons that directly regulate SNS activity.

In hypertensive conditions, RVLM neurons display abnormally increased discharge frequency, leading to increased sympathetic activity and vasoconstriction, thus elevating blood pressure values. RVLM also plays a key role in mediating baroreflex modulation in physiological and pathological conditions. Moreover, RVLM neuronal activity is extrinsically controlled by other cardiovascular regions in the CNS, one of which is the PVN.

The PVN has emerged as one of the major regulators of the coordinated autonomic and endocrine output. PVN projects to both RVLM and the spinal sympathetic intermediolateral nucleus, and PVN neurons stimulation increases RVLM activity and arterial blood pressure. Several PVN neurons that project to RVLM also display an intrinsic auto-rhythmicity, and the discharge frequency correlates closely with sympathetic discharge rate.

The extensive projections of the PVN to central regions (RVLM, area postrema, NTS and intermediolateral nucleus of the spinal cord) indicate that PVN plays a significant role in modulating RVLM activity and sympathetic outflow. The PVN receives input from a large number of regions in the brain, including those associated with osmotic control, appetite, energy metabolism, stress, emotions and other areas that exert effects on BP. Thus, it is clear that the role of the PVN is to integrate inputs from a variety of sources and modify RVLM activity according.

It was also showed that electrolytic lesions of the PVN in SHR elicited an acute reduction of sympathetic activity together with a decrease of blood pressure. Other acute animal studies, performed under general anaesthesia, showed that PVN muscimol injections lowered BP and renal sympathetic nerve activity both in SHR and WKY rats, indicating that this region was tonically active in both animal strains to control BP and peripheral sympathetic activity.

Moreover, the relation of PVN and RVLM neurons to sympathetic control suggests that the spontaneous discharge can be modified through either changes to the intrinsic rate of depolarization or alterations in the balance of excitatory and inhibitory afferent input.

Thus, the present work intended to modulate sympathetic activity in these two central sympathoexcitatory areas – PVN and RVLM - and establish the role of this modulation on sympathetic tone, blood pressure, cardiovascular reflexes function and signalling changes in hypertensive target organs.

For that, a decrease in cellular excitability in PVN and RVLM was promoted in a chronic and conscious animal model of hypertension by the overexpression of a potassium channel induced by a lentivirus. Blood pressure values, autonomic output, the baro- and chemoreceptor function and the molecular signalling in hypertensive target organ were monitored. As a control area was used the medullo-cervical pressor area (MCPA) located in the ventrolateral region of the medullo-cervical junction, which neurons project to the preganglionic sympathetic neurons. It was recently described as an area with high sympathoexcitatory interference in respiratory function, but little action on the cardiovascular system and it doesn’t have any neuronal relay to RVLM or suprabulbar regions.

Results show that chronic overexpression of potassium channels in the PVN and RVLM of conscious unrestrained SHR caused a marked and sustained decrease in blood pressure and sympathetic output as revealed indirectly by a decrease in the power density of the Low frequency (LF) band of systolic blood pressure (SBP). In the PVN, in particular, there is a reversal remodelling of the baro- and chemoreceptor function that approached the normal physiological function. Interestingly, no changes in the baro- and chemoreceptor function were observed with intervention in RVLM, where the sympathetic efferent response is primarily generated. Signalling changes also occurred in hypertensive target organs, heart, kidney and vessels. In fact, the central manipulation that promoted a decrease in blood pressure and sympathetic activity also affected gene expression in target organs, mainly through the up-regulation of angiotensinogen and AT-2 genes in the kidney and down-regulation of AT-1 receptors in the heart.

These results give support to PVN and RVLM role as powerful sites to control BP in neurogenic hypertension and we expect, by identifying the role of these central areas, to provide realistic targets for therapeutic interventions in hypertension.

In conclusion, the present work shows that the intervention on central sympathoexcitatory neurons excitability through the genetic manipulation of K+ channels expression is able to long term alter peripheral blood pressure. This occurs by sympathetic outflow remodelling and by signalling changes that occurred in hypertensive target organs that maintain cardiovascular homeostasis. Our data, from an animal model, give insights into the pathophysiological mechanisms involved in the aetiology of essential hypertension of neurogenic origin and provide novel hypothetical therapeutic interventions at central level of the autonomic nervous system to control sympathoexcitation and functional damage on peripheral organs.

Keywords: Arterial Hypertension, Sympathetic Nervous System, Target Organs, Paraventricular Nucleus of the Hypothalamus (PVN), Rostroventrolateral Medulla (RVLM), Lentiviral Vector (LVV), Baroreflex and Chemoreflex.

CHAPTER 1

CHAPTER 1.

ARTERIAL HYPERTENSION

1.1. INTRODUCTION

I. Defining arterial hypertension

Hypertension is a sustained elevation of systemic arterial pressure that may be evoked by cardiac output increases, however sustained hypertension is due to an increase of total peripheral resistance. Hypertension is a very common abnormality in human subjects and can be produced by a serious of disorders. When, for a long period, an increase in afterload is observed, hypertrophy of the cardiac muscle cells develops. The primary response is the activation of immediate-early genes followed by the activation of a series of fetal genes involved in growing during the fetal period (Opie, 1998; Katz, 2001). In these conditions, O_2 consumption increases not only due to the rise in cardiac work but also due to the further increase of cardiac muscle mass. Therefore, in these patients, any decrease of coronary blood flow has serious consequences and, if the ability of the heart to compensate for the high peripheral resistance is exceeded, the heart can fail (Opie, 1998; Katz, 2001). Hypertensive patients have a higher risk of thrombosis and cerebral hemorrhage and renal failure.

Quantitatively, hypertension is defined by values of systolic blood pressure (SBP) >140 mmHg and/or of diastolic blood pressure (DBP) > 90 mmHg. These values were based on the evidence, from randomized controlled trials, that treatment-induced BP reductions are beneficial for patients (Mancia *et al.*, 2013; James *et al.*, 2014).

According to the 2013 ESH/ESC Guidelines and to the latest report of the Joint National Committee USA (JNC8), a classification of BP values has been made in accordance with the magnitude of the blood pressure (BP) values (see table 1.1). Individuals with values between 130-139 (systolic BP) and 85-89 (diastolic BP) are called pre-hypertensive and

already considered at risk for developing hypertension (Mancia *et al.*, 2013; James *et al.*, 2014). This last category of a *pre-hypertension* category was created by the JNC7 report based on evidences taken from the Framingham study (Vasan *et al.*, 2002; Chobanian *et al.*, 2003). In such long term and particular study, was shown that, in such individuals, the chance of developing hypertension is higher than in those individuals with BP values <120/80 mmHg, termed *normal* blood pressure values (Chobanian *et al.*, 2003). However this terminology was not adopted by the ESH/ESC (Mancia *et al.*, 2013).

Table 1.1. Definitions and classification of blood pressure (BP) levels (mmHg). The BP category is defined by the highest level of BP, whether systolic or diastolic. Isolated systolic hypertension should be graded 1, 2, or 3 according to systolic and diastolic BP values in the ranges indicated. Adapted from ESH/ESC guidelines 2013.

Category of BP values	Systolic BP (mmHg)		Diastolic BP (mmHg)
Optimal	<120	and	<80
Normal	120–129	and/or	80–84
High normal	130–139	and/or	85–89
Grade 1 hypertension	140–159	and/or	90–99
Grade 2 hypertension	160–179	and/or	100–109
Grade 3 hypertension	≥180	and/or	≥110
Isolated systolic hypertension	≥140	and	<90

The 2013 ESH/ESC Guidelines, in common with other guidelines, recommended two distinct BP targets, namely, 140/90 mmHg in low moderate risk hypertensive patients and 130/80 mmHg to high-risk hypertensive subjects which are patients with co-morbidities like diabetes, cerebrovascular, cardiovascular or renal disease. More recently, the European Guidelines on Cardiovascular Disease Prevention recommended a target of 140/80 mmHg for diabetic patients (Perk *et al.*, 2012). In 2013, the European Society of Cardiology Guidelines included a re-evaluation of the target BP upon therapeutics. The recommendation is to target a systolic BP <140 mmHg for most hypertensive patients but for those, aged > 80 years, with systolic BP >160 mmHg was recommends a reduction to

140-150 mmHg, with adjustments according to tolerability in the fragile elderly. A diastolic BP <90 mmHg was recommended for all hypertensive patients, except for diabetic patients, where the 85 mmHg is the target value (Mancia *et al.*, 2013).

Since this hypertension is a progressive cardiovascular syndrome arising from complex and interrelated etiologies it can be classified as essential or secondary arterial hypertension.

I a. Essential hypertension

The primary, essential or idiopathic hypertension can be defined as a rise in blood pressure with no identifiable cause. Is the most common form of arterial hypertension (AHT) affecting about 90 to 95% of patients (Carretero & Oparil, 2000; Bolívar, 2013). It is an heterogeneous disease in which different patients have different causative factors that lead to increased blood pressure values (see II) (Carretero & Oparil, 2000).

The pathophysiology of essential hypertension is an area of research, and until now remains not well understood. It is known that BP regulation is a complex interaction of different cardiac, vascular, renal, neurologic, hormonal, humoral and metabolic mechanisms (Goldberger, 1958; Chopra *et al.*, 2011; Bucher *et al.*, 2013). Therefore, several different pathways may be simultaneously implicated in the development of essential hypertension, such as genetic predisposition, obesity, insulin resistance, excess dietary salt intake, sympathetic over-activation and alterations in sodium homeostasis, renin-angiotensin system, vascular function and inflammation (Lifton *et al.*, 2001; Strazzullo *et al.*, 2003; Geller, 2004; Sowers, 2004; Karppanen & Mervaala, 2006; Savoia & Schiffrin, 2006; Manrique *et al.*, 2009; Savoia *et al.*, 2011; Bucher *et al.*, 2013).

Among the various theories put forward to explain the pathophysiology of essential hypertension, one is the *high blood pressure of neurogenic nature* (Kuchel & Genest, 1977). This hypothesis suggests that a dysfunction in the sympathetic modulation of cardiovascular function is responsible for the hypertensive state, which actively participates in the development and progression of this disease. Other theories refer to

the inability of kidneys to excrete sodium leading to salt and water retention, increased plasma volume, and cardiac output. The overactivity of the renin-angiotensin-aldosterone system has also been referred to increase the secretion of renin that elicits an increase in plasmatic angiotensin II leading to generalized vasoconstriction or to a renal salt and water retention (Cain & Khalil, 2002; Manrique *et al.*, 2009; Santos *et al.*, 2012). Changes in resistance arteries morphological and physical properties together with modifications of endothelial function leads to a decreased vascular relaxation and excessive vasoconstriction promoting significant increases in the peripheral vascular resistance and arterial pressure over time, particularly in aging (Intengan & Schiffrin, 2000; Oparil *et al.*, 2003; Lee & Oh, 2010). The mosaic theory suggests that after a single factor acts as a trigger to raise BP then multiple factors will sustain the increased BP values (Strazzullo et al., 2003).

I b. Secondary hypertension

The secondary hypertension is defined as elevated blood pressure due to an established, identifiable and potentially treatable cause (Mancia *et al.*, 2013). The incidence of secondary AHT is estimated between 5–10% of the overall hypertensive population and is linked to diseases that can affect the kidney, heart, endocrine system, vascular system, lungs and central nervous system (Kaplan, 2005; Chiong *et al.*, 2008). Also, the administration of certain drugs (hormonal contraceptives, antidepressants, corticosteroids), the ingestion of toxic agents (lead, mercury) and due to pregnancy (Nadar & Lip, 2009) can lead to AHT.

Secondary AHT can be identified by symptoms (e.g., flushing and sweating suggestive of pheochromocytoma), examination findings (e.g., a renal bruit suggestive of renal artery stenosis), or laboratory abnormalities (e.g., hypokalemia suggestive of aldosteronism) (Viera & Neutze, 2010).

Renovascular hypertension, the hypertension caused by renal artery stenosis, has two main etiologies - atherosclerosis and fibromuscular dysplasia- the first one with an higher incidence (80-90% of the overall patients) mainly in patients with >65 years (Zeina *et al.*,

2007; Chrysant & Chrysant, 2014). Among all patients with AHT, renal artery stenosis is observed in only 1% to 6% (Simon *et al.*, 1972; Vokonas *et al.*, 1988; Ram, 1997), while the incidence of renal artery stenosis is more than 50% in elderly patients with known atherosclerotic disease (Swartbol *et al.*, 1994; Miralles *et al.*, 1998). However, in young adults (19-39 years), renal artery stenosis is one of the most common secondary etiologies (Elliott, 2008; Viera & Neutze, 2010). The progressive, occlusive process typically narrows the ostium and proximal third of the main renal artery, as well as the nearby aorta. As with all other atherosclerotic vascular diseases, it is found with increasing frequency with advancing age and has the usual associated risk factors (diabetes, dyslipidemia, tobacco use, and history of cardiovascular events) (Elliott WJ, 2008). Fibromuscular dysplasia is a vascular disorder of unknown etiology that has a predilection for the renal arteries in women <40 years causing their narrowing and leading to a decreased renal perfusion (Elliott, 2008; Viera & Neutze, 2010).

In middle-aged adults (40 to 64 years of age), aldosteronism is the most common endocrine cause of secondary AHT (Young, 2007; Viera & Neutze, 2010). Primary hyperaldosteronism is characterized by an overproduction of aldosterone that causes AHT, damage to the cardiovascular system, suppression of plasma renin, sodium retention, and potassium excretion, which leads to hypokalemia (Nyirenda & Padfield, 2007). Aldosterone is also involved in collagen synthesis, producing vascular remodeling and myocardial fibrosis in a process that is independent of its effect on arterial blood pressure (Bunda *et al.*, 2007; Abad-Cardiel *et al.*, 2013). Thus, it is important to identify patients with primary hyperaldosteronism, since an increase in plasma aldosterone is associated with negative cardiac and vascular effects and a greater risk of suffering a cardiovascular event (Milliez *et al.*, 2005; Abad-Cardiel *et al.*, 2013).

Other causes of secondary AHT in middle-aged adults are: obstructive sleep apnea, pheochromocytoma and the Cushing syndrome (Viera & Neutze, 2010). Thyroid dysfunction is also a cause of secondary AHT, mainly in young adults. Thyroid hormones affect cardiac output and systemic vascular resistance impacting blood pressure values. Hypothyroidism can cause an elevation in diastolic blood pressure, whereas

hyperthyroidism can cause an isolated elevation of systolic blood pressure, leading to a widened pulse pressure (Klein & Danzi, 2007).

In children (0 to 18 years of age) with AHT, up to 85% have an identifiable cause, most often renal parenchymal disease (Roccella EJ *et al.*, 2004; Viera & Neutze, 2010). In this age group, such renal pathology includes glomerulonephritis, congenital abnormalities, and reflux nephropathy. Coarctation of the aorta is the second most common cause of hypertension in children, and is two to five times more common in boys than in girls (Brickner *et al.*, 2000). Rarely, mild cases of coarctation have occurred in adults (Viera & Neutze, 2010).

In the coming years, some forms of secondary hypertension tend to come more prevalent. Renovascular disease due to atherosclerosis is an example, since it is associated with greater longevity and with aging population. Likewise, primary aldosteronism by changes in screening paradigms and obstructive sleep apnea syndrome, due to the rise in obesity (Thomopoulos et al., 2011; Abad-Cardiel et al., 2013; Fleg et al., 2013) are increasing their prevalence among hypertensive patients.

II. Epidemiology of arterial hypertension

Arterial Hypertension (AHT) is considered one of the major risk factors for cardiovascular and cerebrovascular diseases. Affects approximately one billion people worldwide and is estimated that could kill nine million people each year (World Health Organization, 2013).

A study from Kearney *et al.*, showed that 26,4% of the adult population in 2000 had AHT (26,6% of men and 26,1% of women), and 29,2% were projected to have this condition by 2025 (29,0% of men and 29,5% of women). The estimated total number of adults with AHT in 2000 was 972 million; 333 million in economically developed countries and 639 million in economically developing countries. The number of adults with hypertension in 2025 was predicted to increase to 1,56 billion (Kearney *et al.*, 2005).

The ESH/ESC 2013 Guidelines for the management of arterial hypertension recognize that the prevalence of hypertension is underestimated, but appears to be about 30–45% of the general population, with a steep increase with ageing (Mancia *et al.*, 2013) .

In Portugal, AHT is highly prevalent, since it is estimated that 42.1% of the Portuguese adult population, aged 18 to 90 years, have hypertension. In a study that included a total of 5023 adults, the age-specific prevalence of AHT in the three age-groups studied - under 35 years, 35-64 years, and over 64 years - was 26.2%, 54.7% and 79% in men and 12.4%, 41.1% and 78.7% in women respectively (De Macedo *et al.*, 2007) (Fig. 1-1).

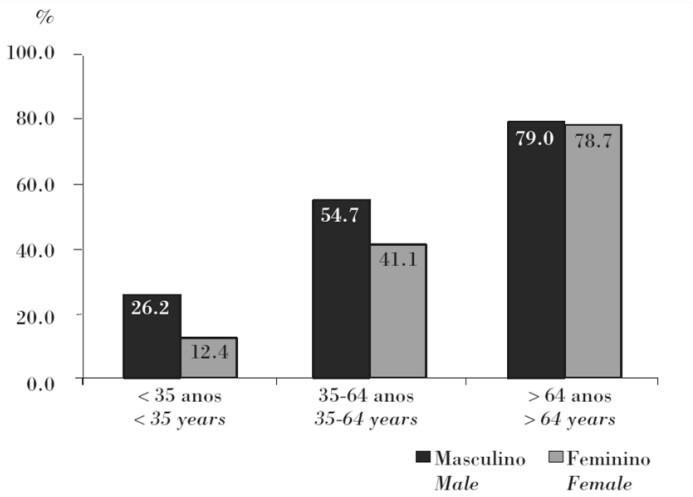


Figure 1-1. Prevalence of AHT in Portugal by sex and age group (<35, 35-64, and >64 years). Extracted from De Macedo et al., 2007.

Analysis by region revealed that the North region had the lowest prevalence of hypertension (33.4%) and the Alentejo had the highest (49.5%) (Fig.1-2).

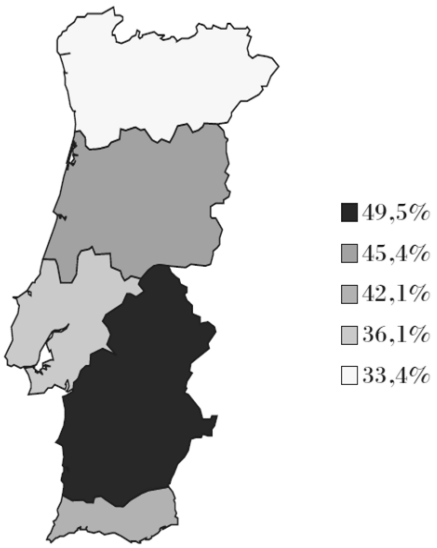


Figure 1-2. Prevalence of AHT in Continental Portugal by region. The country was divided into 5 regions. Black indicates an higher prevalence and white a lower prevalence. Extracted from De Macedo et al., 2007.

In this study, was also found that hypertension was sub-diagnosed as among hypertensive subjects, only 46.1% were aware of their high blood pressure. Thirty-nine percent of the individuals included in the study were taking antihypertensive medication and 11.2% had their blood pressure controlled (De Macedo *et al.*, 2007). From this study, it is evident that AHT is an important public health challenge in Portugal as well as worldwide because its complications, including cardiovascular, cerebrovascular, and renal diseases, are the major causes of morbidity and mortality. Therefore, prevention, detection, treatment, and control of this condition should receive high priority (Kearney et al., 2005; Messerli et al., 2007).

III. Risk factors for essential hypertension

There are several risk factors that may contribute to the development of the essential hypertension including genetic predisposition, environmental factors and lifestyle.

Results from family studies suggest an hereditary predisposition to the development of essential hypertension, with the heritability estimated to vary between 35% and 50% in the majority of studies. Two Genome-Wide Association Studies (GWAS) have shown 13 loci associated with BP/AHT, and an extensive meta-analysis of GWAS data, with a total sample size of nearly 200 000 people of European descent, have identified 16 novel loci associated with systolic BP and diastolic BP (Levy *et al.*, 2009; Newton-Cheh *et al.*, 2009; Ehret *et al.*, 2011). Indeed, a total of 29 single nucleotide polymorphisms associated with systolic and/or diastolic BP (Mancia et al., 2013). It also been showed that these polymorphisms are associated with the incidence of coronary events and stroke (Ehret *et al.*, 2011). Identifying genes associated with high blood pressure advances our understanding of blood pressure regulation and highlights the potential drug targets for the prevention or treatment of hypertension (Levy *et al.*, 2009). However, the genetic heterogeneity, the imprecision in measuring specific phenotypes and the variability of the sampling methods contribute to weaknesses and inconsistencies between reported studies (Williams *et al.*, 1994).

Environmental causes of hypertension include poor diet (especially those that include large quantities of salt), smoking, heavy and regular use of alcohol and lack of physical activity (Chobanian *et al.*, 2003; Ong *et al.*, 2008). In fact, the development of AHT appears to be more common in heavy salt consumers. However, on a similar dietary salt ingestion, some individuals develop hypertension while others do not with the probability to develop hypertension dependent on the individual weight of the hypertension adjunctive factors (Juan J. Bolívar, 2013). Hence, in general terms, a controlled diet can significantly reduce blood pressure (Appel *et al.*, 1997). Other factors that lead to increased BP are insulin resistance, low potassium intake and low calcium intake (Carretero & Oparil, 2000).

It is well established that the incidence of AHT is higher among smokers, alcoholics and people with a stressful life (Arkwright *et al.*, 1982; Lee *et al.*, 2001), which is a reason why AHT predominates in industrialized societies and it is included in the so-called *diseases of civilization*. The coincidence of two or more risk factors - overweight, high salt intake, smoking, alcohol and stress - significantly increases the possibility of developing arterial hypertension (Appel *et al.*, 2003; Dickinson *et al.*, 2006; The Trials of Hypertension Prevention Collaborative Research Group, 1997).

Several reports published controversial results about the association of physical activity with risk of hypertension. In fact, a meta-analysis of prospective cohort studies was performed to investigate the effect of physical activity on hypertension risk and the results suggested that there was an inverse dose–response association between levels of recreational physical activity and risk of hypertension, whereas there was no significant association between occupational physical activity and hypertension (Huai *et al.*, 2013). Individuals with a sedentary lifestyle have 30% greater risk of developing hypertension compared with active individuals (Paffenbarger *et al.*, 1991).

It was found that the AHT is more common among obese people, settling up a direct link between the significant excess weight and the disease. Sedentary lifestyles, changes in dietary habits, among others, contribute to the development of obesity, and this consequently leads to the onset of the disease (De Macedo *et al.*, 2007). In addition, in patients with hypertension associated with obesity, there is an increase in renal

sympathetic outflow when compared to obese people with normal BP. It is, therefore, considered that the obesity-related hypertension has an important neurogenic component (Tuck, 1992).

Blood pressure values have a physiological linear increase with age. With an increasingly aging population, age is becoming the major risk factor for arterial hypertension (Wolf-Maier *et al.*, 2003). About 60% of the elderly population in the world, aged > 60 years has hypertension (Bobrie & Potter, 2002). In older people, the AHT appears mainly as a result of an increase in SBP, being named isolated systolic hypertension, whereas in young subjects, the onset of AHT is mainly due to the raise in DBP (Sobotka *et al.*, 2011). The change in blood pressure with age is manly associated with increased arterial stiffness, which results in a progressive replacement of elastin by collagen in the walls of large arteries also referred to as arteriosclerosis. This process leads to dilation and stretching of the aorta and its branches through fibrosis and hypertrophy of arterial muscle (Izzo *et al.*, 2000).

Even so, these risk factors cannot be considered in isolation. Thus, an effective strategy for the treatment of hypertension should involve either pharmacological therapeutics or therapeutics of other natures complemented with lifestyle changes.

IV. Target organs damage

The target organs damage due to chronic arterial hypertension is a major cause of cardiovascular morbidity and mortality. The classic manifestations of hypertensive end organ damage include vascular and hemorrhagic stroke, retinopathy, coronary heart disease/myocardial infarction and heart failure, proteinuria and renal failure and in the vasculature, atherosclerotic change including the development of stenosis and aneurysms (Schmieder, 2010; Table. 1.2).

Table 1.2. End organ damage in arterial hypertension. Adapted from Schmieder, 2010.

End organ damage in arterial hypertension	
Vasculopathy • Endothelial dysfunction • Remodelling • Generalized atherosclerosis • Arteriosclerotic stenosis • Aortic aneurysm	**Cerebrovascular damage** • Acute hypertensive encephalopathy • Stroke • Intracerebral haemorrhage • Lacunar infarction • Vascular dementia • Retinopathy
Heart disease • Left ventricular hypertrophy • Atrial fibrillation • Coronary microangiopathy • CHD, myocardial infarction • Heart failure	**Nephropathy** • Albuminuria • Proteinuria • Chronic renal insufficiency • Renal failure

The sympathetic nervous system (SNS) overactivity in essential AHT is not only important for the initiation and maintenance of elevated blood pressure (BP) values, but also for the progression of organ damage in both humans and animal models (Mancia *et al.*, 1999; Rahn *et al.*, 1999; Morise *et al.*, 2000; Kasparov & Teschemacher, 2008; Fisher & Fadel, 2010; Tan *et al.*, 2010). Progression of the disease is strongly associated with functional and structural abnormalities that damage the heart, kidneys, brain, vasculature and other organs and the manifestations due to chronic elevation of BP are a direct consequence of changes in these organs (Giles & Sander, 2005). Hence, the reduction of BP when there is target organ damage or if the functional precursor of the target organ damage is present and still reversible, generally reduces the risk for cardiovascular events.

IV a. Effect in the heart

Hypertensive heart disease has been defined as the response of the heart to the afterload imposed to the left ventricle by the progressive increasing of BP and total peripheral resistance by the hypertensive vascular disease (Rafique, 1993). In particular, hypertensive heart disease is characterized by altered coronary hemodynamics and reserve, cardiac dysrhythmias, left ventricular hypertrophy and enlargement, ventricular

fibrosis, systolic/diastolic dysfunction and cardiac failure. There are many factors that contribute jointly to develop the disease. These are genetic determinants, environmental risk factors and hemodynamic and non-hemodynamic mechanisms (Crawford *et al.*, 2004) (Fig. 1-3).

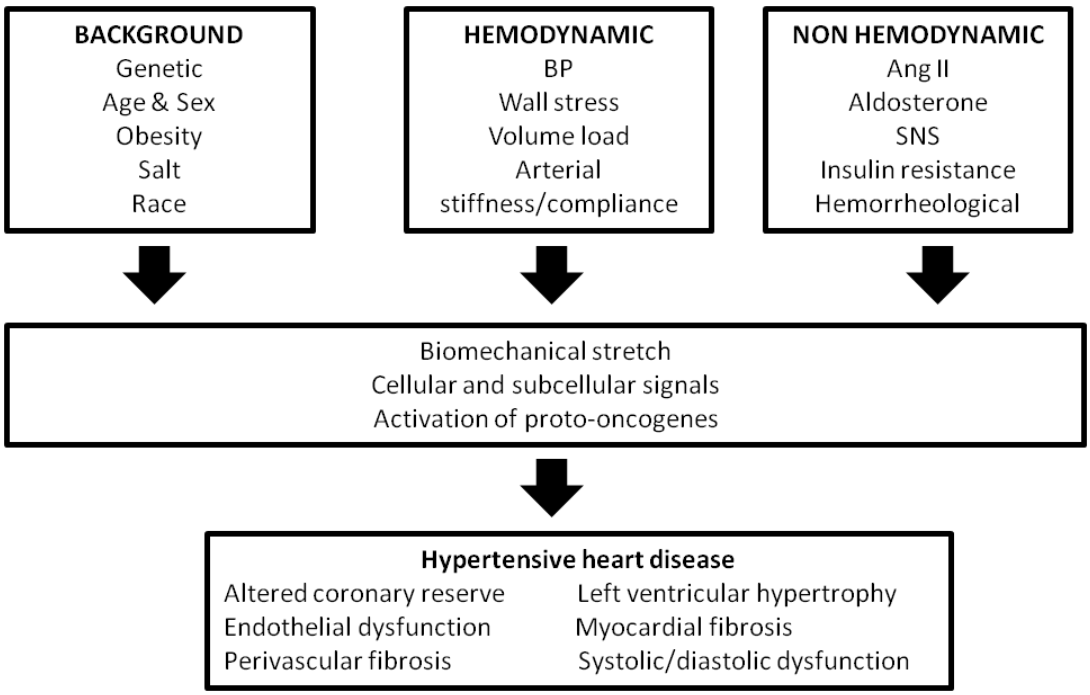


Figure 1-3. Determinants of hypertensive heart disease. Adapted from Crawford et al., 2004.

However, the hemodynamic factors do not act isolated but in conjunction with non-hemodynamic factors - age, race, obesity, salt intake, insulin resistance together with neuroendocrine (angiotensin II, aldosterone, endothelin) and hemorrheologic factors (blood viscosity and plasma volume) (Crawford *et al.*, 2004). All of them undergo a complex and interrelated degenerative/adaptive process in order to answer to the persistent increase of blood pressure (see table 1-3 for details).

The hemodynamic load, either by an increase in peripheral resistance or in cardiac output, is the basic initial stimulus to begin the sequence of events that lead to heart disease in hypertension. Early hemodynamic changes in hypertension often include increased or inappropriately high resting cardiac output, especially in obese patients, whereas vascular resistance tends to be normal or inappropriately high in proportion to the chronic level of blood pressure (FREIS, 1960; Messerli, 1982; Izzo *et al.*, 2008). In non-

obese subjects with established essential hypertension the hemodynamic pattern seen at rest, most often, is normal blood flow with elevated vascular resistance (Messerli *et al.*, 1983; Izzo *et al.*, 2008) along with increased arterial stiffness (Mitchell, 2004).

Table 1-3. Pathogenetic processes underlying cardiac damage in hypertension. Extracted from Crawford et al., 2004.

Pathogenetic processes underlying cardiac damage from hypertension	
Neurohormonal	Activation of the rennin-angiotensin-aldosterone system Enhanced adrenergic activity Increased production or reduced degradation of biomolecules (eg angiotensin, cytokines)
Hemodynamic	Increased peripheral resistance Increased wall stress Decreased coronary reserve
Vascular	Endothelial dysfunction Vascular remodelling Decreased vascular compliance Exaggerated vascular reactivity Coronary and peripheral vascular atherosclerosis
Myocardial	Left ventricular remodelling Foetal gene expression Myocite hypertrophy Alterations in extracellular matrix

The variation in performance between a normal and an hypertrophied heart may be quite marked. For example, Beznak found an initial drop in cardiac output and cardiac reserve following acute experimental coarctation of the aorta in the rat (Beznak, 1958). However, in animals with chronic coarctation and the resulting myocardial hypertrophy, the cardiac output was normal. It appears, therefore, that there are at least three possible mechanisms by which the left ventricle might adjust to an elevated peripheral resistance: I) an increase in residual volume, and, hence, diastolic fiber length; II) augmentation of intrinsic *contractility* of the myocardial fibres independent of fibre length, and III) hypertrophy (Freis, 1960).

IV b. Effect in the vascular system

The increase in pulse pressure in the presence of a normal cardiac output indicates loss of large artery distensibility (Freis, 1960; Mitchell *et al.*, 2008). This appears to be brought about in chronic hypertension by passive distension, accelerated atherosclerosis and, occasionally, by degenerative changes in elastic tissue. As a result of these changes pulsatile pressures are transmitted further than normally into the peripheral circulation.

Abnormalities of the small vessels have been described in hypertensive patients. In the conjunctivae, these abnormalities include diminished number of visible capillaries (189), abnormally thin and tortuous capillaries (Lack & Adolph, 1949; Lee & Holze, 1951; Landau & Davis, 1957; Freis, 1960), constricted terminal arterioles and metarterioles which exhibit increased reactivity to topical epinephrine (Lee & Holze, 1951; Jackson, 1958), and increased intermittency of capillaries (Jackson, 1958). Any one of these changes is seen occasionally in normal individuals, but the presence of all features is characteristic of hypertension (Lee & Holze, 1951), and they were seen with much greater frequency in hypertensive patients (Landau & Davis, 1957).

One of the most serious complications of arterial hypertension is at the level of the arteries. The persistent elevation of blood pressure tends to cause lesions in the walls of arteries, leading to the hardness of blood vessels (arteriosclerosis) and promoting fat deposits in the vessels which leads to the formation of atherosclerotic plaques (atherosclerosis) (Susic, 1997). This promotes a decrease in blood flow as well as an increased risk of bleeding.

Regional blood flows are generally not impaired in hypertension although blood flow reserve may be reduced in circumstances requiring increased blood flow (e.g. exercise). Oxygen consumption is also normal (Izzo *et al.*, 2008). Therefore, in chronic human AHT, with the exception of the kidney, where flow usually is slightly to moderately reduced, cerebral, coronary and hepatic blood flows are all within the normal range (Freis, 1960).

IV c. Effect upon renal function

Scientific data demonstrated that systemic hypertension has a major role in the progressive loss of renal function (Ruilope, 2008) as hypertension is frequently associated with fibrinoid deposition in the renal glomeruli and proteinuria (Cohuet & Struijker-Boudier, 2006). Renal injury occurs when the preglomerular autoregulatory mechanism is insufficient to maintain flow and pressure in the kidney (Griffin *et al.*, 2003). Loss of renal autoregulation with glomerular hypertrophy, hyperfiltration and focal segmental glomerulosclerosis is now recognized to contribute significantly to nephrosclerosis which functional and morphological abnormalities are intimately linked to the overactivation of renin-angiotensin system (Volpe et al., 2002). Uncontrolled AHT is a risk factor for developing chronic kidney disease and is associated with a more rapid progression of the disease. Progressive renal disease can exacerbate uncontrolled AHT due to volume expansion and increased systemic vascular resistance. Therefore, several guidelines discussed the importance of lowering blood pressure to slow the progression of renal disease and reduce cardiovascular morbidity and mortality (Mancia *et al.*, 2013; James *et al.*, 2014). It is also known that chronic kidney disease is a risk factor for cardiovascular disease, and that a reduced glomerular filtration rate (GFR) and albuminuria are associated with an increase in cardiovascular and all-cause mortality (Rashidi *et al.*, 2008; Matsushita *et al.*, 2010).

IV d. Effect in the brain

AHT causes changes in cerebral circulation leading to four major cerebrovascular illnesses such as ischemic stroke, lacunar infarction, hypertensive encephalopathy, and hypertensive brain haemorrhage are associated with AHT (Lee, 1989). In fact, the cerebral circulation is susceptible to damage by sudden increases in arterial pressure. During episodes of acute, severe hypertension, cerebral vessels dilate passively and there is a "break- through" of autoregulation (Baumbach & Heistad, 1988). An hypertensive crisis may cause ischemic stroke, due to a thromboembolic event or a hemorrhagic stroke (Johansson, 1999). The majority of diagnosed stroke cases (80%) are due to ischemia and infarction secondary to occlusive disease of the small and medium size cerebral arteries

(Lewington *et al.*, 2002). Regarding in particular the cerebral circulation, it has been suggested that there are at least two distinct pathological processes that play a role in AHT-induced cerebrovascular damage, separated on the basis of vessel size and type (Doyle, 1983). The evidence supporting this hypothesis is histological, since in the larger intracranial and extracranial arteries of hypertensive patients, complex atherosclerotic lesions are the main finding. In smaller vessels, hyaline necrosis, the formation of microaneurysms, and lipid degeneration of the arterial wall are more commonly observed (Dinsdale, 1978; Conomy, 1980). AHT can induce rupture of diseased large and small arteries, possibly as a result of localized cellular degeneration within the arterial wall (Takebayashi & Kaneko, 1983).

Hypertension may also cause damage in specific regions of the brain (Conomy, 1980; Dinsdale, 1983). As an example, hypertensive haemorrhage occurs predominantly in the cerebral hemispheres, whereas the arterial lesions in hypertensive encephalopathy occur predominantly in the brain stem and basal ganglia and to a lesser extent, in the cerebral hemispheres (Conomy, 1980). Damages in cerebral vasculature may be also associated with at least one form of dementia, defined as an irreversible and usually progressive loss of cognitive and intellectual functions (Lee, 1989). A significant impairment of attention span and vigilance was reported in untreated hypertensive patients compared with the normotensive patients (Boller *et al.*, 1977). Therefore, high blood pressure is indirectly an important risk factor for cognitive decline later in life.

As mentioned above, autoregulatory mechanisms are altered in chronic hypertension. Some of these changes are compensatory and beneficial, since the normotensive patients are more susceptible to hypertensive encephalopathy from sudden pressure rise than hypertensive patients (Lee, 1989). Others changes are not beneficial and predispose the individual to ischemia and stroke. Blockade of angiotensin II formation or bradykinin degradation, with ACE inhibitors, prevents and reverses these alterations, improves tolerance to hypotension (Torup *et al.*, 1993) and protects against focal cerebral ischemia in spontaneously hypertensive rats (SHR) (Hajdu *et al.*, 1991; Fujii *et al.*, 1992). In a similar way, acute administration of candersartan - a potent AT1 receptor antagonist - in SHR shifts their cerebrovascular autoregulatory response, in the direction of lower BP

(Vraamark *et al.*, 1995). In summary, chronic hypertension is clearly damaging the cerebral vasculature and has been termed the "preeminent precursor of stroke"(Lee, 1989).

V. Signalling in hypertension

Evidence from clinical and basic research has demonstrated that the functional changes evoked by a primary increase of sympathetic activity and blood pressure elicit signalling changes which are intimately implicated in the regulation of adaptive events in hypertension including those related to peripheral artery resistance, vasodilation, contraction and vascular tone. There is an extensive bibliography on this subject but only those directly related to the core of the present work will be mentioned. These include natriuretic peptides, angiotensin II and other elements of the renin angiotensin aldosterone system (RAAS), endothelin-1, redox and mitochondrial factors, all of them being particularly involved in essential hypertension pathogenesis.

V a. Renin-angiotensin-aldosterone system (RAAS)

Angiotensin II (Ang II) is the major peptide hormone of the RAAS having a critical role in the control of cardiovascular homeostasis, including mediation of peripheral artery resistance, vasodilation, contraction, and vascular tone. Ang II function is mediated by the AT1 and AT2 receptors, the first type activating several cytoplasmic signalling pathways, which contribute to vascular remodeling by inducing hypertrophy, hyperplasia and migration of vascular smooth muscle cells (SMCs) together with endothelial dysfunction (Touyz & Schiffrin, 2000). Ang II through the AT1 receptor activates small GTP binding proteins that appear to play important roles in mediating cardiovascular remodeling induced by Ang II, in particular RhoA which is a regulatory factor of cytoskeletal dynamics, transcription, cell cycle progression and cell transformation (Yamakawa *et al.*, 2000; Ohtsu *et al.*, 2006b). On the other hand, RhoA and ROCK, its downstream effector, seem to exert a negative regulatory effect on eNOS gene expression by inducing a destabilization of eNOS mRNA and a positive regulatory effect on ET-1 transcription favoring vascular occlusion (Barandier *et al.*, 2003). The pair RhoA-ROCK is also involved in

the regulation of the endothelial barrier dysfunction and enhanced contraction of vascular smooth, the later due to an increase of myosin light chain phosphorylation (Lee *et al.*, 2004). Ang II through AT1 receptor may, also, induce vascular smooth muscle hypertrophy through the trans-activation of epidermal growth factor receptor (EGFR, ErbB-1; HER1) leading to c-Fos induction and smooth muscle cells proliferation (Eguchi & Inagami, 2000; Eguchi *et al.*, 2001; Kagiyama *et al.*, 2002; Ohtsu *et al.*, 2006a) (Fig. 1-4). By regulating several non-receptor tyrosine kinases, in particular, a proto-oncogene tyrosine-protein kinase (Src) which is activated by reactive oxygen species as well as c-fos and c-myc, Ang II is also able to control cell growth and proliferation (Berk & Corson, 1997).

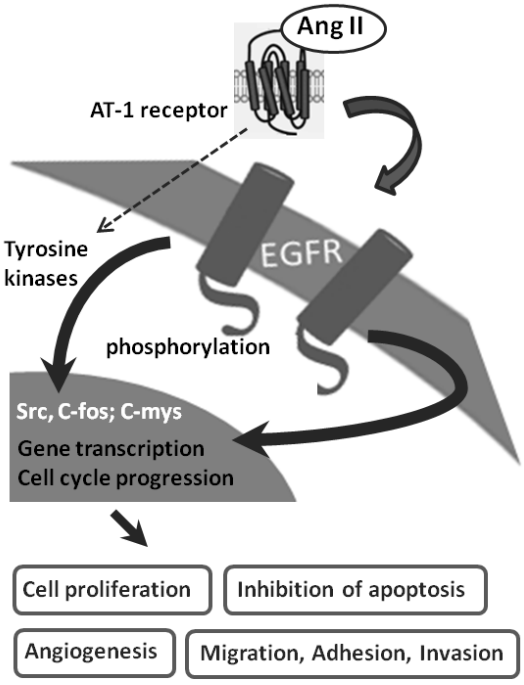


Figure 1-4. Diagram showing the general interactions between AngII and other co-factors to promote target organ damage in hypertension. The mechanism of EGFR transactivation involves a metalloprotease which when inactivated blocks Ang II-stimulated hypertrophy. Adapted from EGFR signalling pathway.

Ang II together with aldosterone and endothelin plays a central role in the remodeling of extra cellular matrix (ECM) in hypertension which is characterized by a fibroblastic activation and increased expression of collagen, fibronectins and integrins which leads to an extensive myocardial fibrosis and myocardial stiffness (Booz & Baker, 1995; Berk *et al.*,

2007; Marín-García *et al.*, 2011). This condition that can be facilitated by the activation of RAAS and the increased activation of transforming growth factor b1 (TGF-b1) by renin and pro-renin (Nguyen, 2006) leads to the recruitment of vascular smooth cells, monocytes, and fibroblasts and stimulates a genetic program of wound repair and extracellular matrix (ECM) deposition, leading to perivascular fibrosis and amplification of the profibrotic state (Iaccarino *et al.*, 2004). In physiological conditions, Ang II promotes the association of scaffolding proteins leading to focal adhesion and ECM formation. Thus, changes in ECM leads to modification in gene expression associated with hypertrophy and contractile dysfunction (Iaccarino *et al.*, 2004; Marín-García *et al.*, 2011). The Ang II effects described above are dependent on Ang II availability, which, in turn, depends from a cascade of reactions and activations, which have renin as a precursor.

Since there is a positive correlation between the angiotensinogen levels and blood pressure values (Dzau & Ingelfinger, 1989; Jeunemaitre *et al.*, 1992; el-Dahr *et al.*, 1993; Nakamura & Johns, 1994; Lodwick *et al.*, 1995; Kirby *et al.*, 1996) several studies, both in animals and human subjects, have suggested that abnormalities in the regulation of angiotensinogen gene expression may be involved in the pathogenesis of AHT (Jeunemaitre *et al.*, 1992; Kimura *et al.*, 1992; Fukamizu *et al.*, 1993; Caulfield *et al.*, 1994; Yang *et al.*, 1994). In fact, the angiotensinogen-deficient mouse shows low values of arterial blood pressure, showing the impact of angiotensinogen in the maintenance of BP and in the development of AHT (Pratt *et al.*, 1989; Tanimoto *et al.*, 1994).

Renin is mainly produced through an enzymatic activation of pro-renin at granular cells of the renal juxtaglomerular apparatus. There is evidence of a relation between renal sympathetic nerve activity and the production and release of renin which gene expression is increased in SHR, an animal model of hypertension (Antonaccio *et al.*, 1984; Samani *et al.*, 1989; Nakamura & Johns, 1995). In fact, renal denervation in the rat blunted the increase in renal renin mRNA after long-term ureteral obstruction (el-Dahr *et al.*, 1991). Similarly, renal renin mRNA levels were lower in denervated than innervated kidneys (Page *et al.*, 1992) showing that tonic activity in the renal nerves could elevate renin gene expression. To induce the conversion of angiotensinogen to angiotensin II, renin binds to an ATPase H(+)-transporting lysosomal accessory protein 2, or the prorenin receptor (Nguyen et al., 2002; Nguyen, 2006). The pro-renin receptor activation encompasses two types of actions: one related directly to all processes that involve the final production of

Ang II and another, which reveals a specific function for renin and pro-renin independent of Ang II production receptor (Nguyen et al., 2002; Nguyen, 2006). In the first case, pro-renin receptor activation increases the enzymatic activity of renin accelerating the production of Ang I on the cell surface and induces the non-proteolytic activation of pro-renin contributing to Ang I production. In the second case, it involves the activation of signalling pathways leading to induce DNA synthesis and stimulation TGF-b (Nguyen et al., 2002; Nguyen, 2006) which participates in a biochemical cascade leading to smooth cells hypertrophy and fibrosis (Iaccarino *et al.*, 2004).

The angiotensin-converting enzyme (ACE) cleaves Ang I and bradycinin thus interacting simultaneously with RAAS and the kallikrein-kinin system, that is implicated in many physiological and pathological processes, including blood pressure regulation, sodium homeostasis, inflammation and cardioprotective effects of preconditioning (Campbell, 2001). Thus, a disturbance of ACE activity may induce vasoconstriction and salt retention.

Aldosterone is another component of the RAAS system that modulates partially the vascular tone by binding to a mineralocorticoid receptor promoting the upregulation of Ang II receptors under conditions where the availability of endothelial NO is reduced. Moreover, an aldosterone excess promotes collagen deposition in blood vessels, enhancing vascular remodelling and peripheral blood monocytes and vascular smooth cells are both influenced by aldosterone to produce ROS. Interestingly, is that activated aldosterone mineralocorticoid receptor can activate signalling pathways in vascular smooth cells to which the cross-activation EGFR- and Src-dependent signalling pathways is needed (Callera *et al.*, 2005; Grossmann *et al.*, 2005; Ishizawa *et al.*, 2005).

V b. Endothelial signalling

In hypertension, endothelium suffers functional and structural modifications losing its normal protective function, which includes relaxation of the vascular smooth muscle and a number of antiatherosclerotic actions. Endothelial dysfunction is characterized by significance decrease of NO availability due to an increase breakdown of NO by reactive oxygen species (ROS) and a reduction of NO synthesis due to a decreased eNOS activity. These alterations elicit a impairment of endothelial function characterized, not only, by a decreased ability of the endothelium to vasodilate but also by an increased ability to

vasoconstric when activated by several contracting factors like endothelin-1, ang II, cyclooxygenase (COX)-derived prostanoids (thromboxane A2, prostaglandin H2) and ROS (Fig. 1-5; Marín-García *et al.*, 2011).

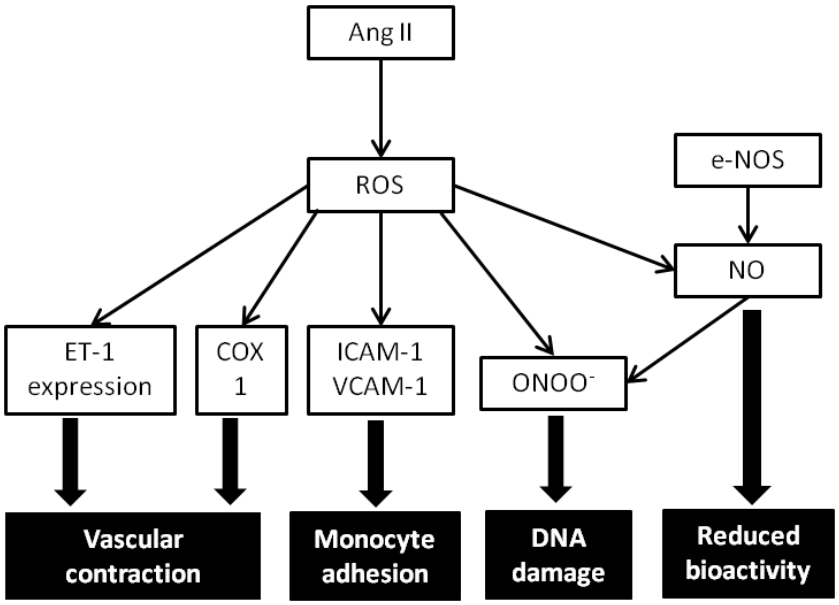


Figure 1-5. Angiotensin II related signalling pathways involved in endothelial dysfunction. COX1 cyclooxygenase 1, eNOS endothelial nitric oxide synthase, ICAM-1 intercellular adhesion molecule-1, *VCAM-1* vascular cell adhesion molecule-1, ONOO− peroxynitrite, ROS reactive oxygen species, eNOS endothelial nitric oxide synthase. Adapted from Marín-García et al., 2011.

ROS are not only released at the endothelial level but also at smooth muscle and inflammatory cells within the arterial wall. All these sources of ROS concur to the accumulation of peroxynitrites and NO destruction and decreased bioavailability that could be, at least partially, restored by the administration of antioxidants like vitamin C (Taddei *et al.*, 1998).

Endothelial derived constrictors factors (EDCF) are COX-derived products released in response to shear stress or to acetylcholine stimulation (Lüscher & Vanhoutte, 1986; Huang *et al.*, 2000; Félétou *et al.*, 2011) and endothelin (Verhaar *et al.*, 1998; Barton, 2000; Félétou *et al.*, 2011; Ohkita *et al.*, 2012; Kaoukis *et al.*, 2013; Moorhouse *et al.*, 2013). The former, with a most relevant role in the physiopathology of endothelium-dependent contraction, are thromboxane A2 and prostaglandin H2. They diffuse to the vascular smooth muscle cells and by acting on thromboxane receptors (TP) (Vanhoutte *et*

al., 2005) induce vasoconstriction which can be reversed by COX inhibitors and TP antagonists (Taddei *et al.*, 1993; Virdis *et al.*, 2007; Félétou *et al.*, 2011). Endothelin presents a dual and opposite effect depending of the type of receptors to which binds as ET-A receptors stimulate vascular contraction whereas ET-B receptors mediate NO release promoting vasodilation. In the hypertensive endothelial dysfunction ET-A receptors are impaired thus the observed endothelial constriction is due to ET-B receptors activation not regulated by ET-A mediated vasodilation (Penna *et al.*, 2006; Kohan *et al.*, 2011; Ohkita *et al.*, 2012; Kaoukis *et al.*, 2013; Moorhouse *et al.*, 2013)

V c. Natriuretic peptides

The major natriuretic peptides (NP) signalling effects upon blood pressure regulation are the relaxation of vascular smooth cells and the antagonism of renal renin-angiotensin-aldosterone system despite can be also involved in the earlier cardiomyocite differentiation as well as in the hypertensive cardiac hyperthrophy (Harris *et al.*, 1987; Calderone *et al.*, 1998; Gambaryan *et al.*, 1998; Ellmers *et al.*, 2002; Lumsden *et al.*, 2010; Hayek & Nemer, 2011; Kohan *et al.*, 2011).

This peptide family which acts through NPR-A, NPR-B and NPR-C receptors, includes three structurally related peptides: atrial natriuretic peptide (ANP) produced in the atria, a ventricular natriuretic peptide (BNP) produced by the ventricles and a C-type natriuretic peptide (CNP) which does not show direct natriuretic activity but is rather a potent arterial and venodilator and a chrono and inotropic agent by its selective agonism for the NPR-B and NPR-C receptors to which it is the sole ligand (Lumsden *et al.*, 2010; Hayek & Nemer, 2011).

There is a close association between natriuretic peptides and the geometric arrangement of the sarcomeres of cardiac muscle fibers under stress. Upon a response to an increase in systolic load, the new sarcomeres will be arranged in parallel to provide an increase of cardiac muscle fibre and a concentric pattern of cardiac hyperthrophy characterized by an increase in ventricular wall thickness and reduction of chamber volume is observed. However, when the load stress is persistent, like in the great majority of hypertensive patients, the diastolic dysfunction will induce the formation of new sarcomeres

rearranged in a series pattern promoting an eccentric pattern of cardiac hypertrophy characterized primarily by an increase in chamber volume and a modest increase in ventricular wall thickness. All these sarcomeric changes underline rearrangements at cellular and molecular level. During heart development, the proliferative ability of the cardiomyocites is lost and important qualitative changes in the expression of the cardiac-specific genes will occur (Zak, 1974; Nadal-Ginard & Mahdavi, 1989). These include downregulation of fetal genes encoding fetal contratile proteins like skeletal α-actin and β-myosin heavy chain together with a decrease expression of ANP-mRNA in the myocardium (Nadal-Ginard & Mahdavi, 1989). In animal models, the rise of afterload lead to an increase of cardiac mass and to the expression of genes reminiscent of the embryonic heart which include those related to contractile proteins and ANP-mRNA (Mercadier *et al.*, 1981; Schiaffino *et al.*, 1989). Coincident with these changes is the downregulation of the adult specific gene sarcoplasmic reticulum Ca^{2+}ATPase (SERCA2) (de la Bastie *et al.*, 1990).

The adaptive response which is the change from α to β-myosin heavy chain leads to a decreased myosin ATPAse activity and contractile response for the heart (Mercadier *et al.*, 1981) thus, reducing the usage of ATP in the presence of a greater work load and oxygen demand (Katz, 1990). The increase of ANP-mRNA observed in both types of cardiac hyperthrophy could represent a counter regulatory mechanism due to the anti-hyperthrophic and antifibrotic actions of natriuretic peptides (Harris *et al.*, 1987; Calderone *et al.*, 1998; Gambaryan *et al.*, 1998; Ellmers *et al.*, 2002; Lumsden *et al.*, 2010; Hayek & Nemer, 2011; Kohan *et al.*, 2011). SERCA2 mRNA down-regulation is in part responsible for abnormal Ca^{2+} handling at the sarcoplasmatic reticulum and the following impairment of myocardial relaxation observed in concentric hyperthrophy (de la Bastie *et al.*, 1990). However, if the re-expression of ANP-mRNA could be considered a conserved molecular event of pathological cardiac hyperthrophy, the changes between the different types of contractile proteins seem to depend on the hemodynamic stimulus as in animal studies, where the primary stimulus for hyperthrophy was increase of volume rather than the increase in pressure, were not observed modifications on the expression of β-myosin, α-actin or the reciprocal down-regulation of SERCA2 (Calderone *et al.*, 1998). This different behaviour indicates may indicate that at mRNA level these genes can be

regulated differently according to the characteristics of the stimulus and that the expression of ANP-mRNA could be secondary and dependent on the hyperthrophic growth of the cardiomyocites. This contrasts with the regulation of α-actin, β-myosine and SERCA2 mRNA which seems to be less conserved events on the hypertrophic process and more stimuli specific.

V d. Redox and mitochondrial signalling

Reactive oxygen species (ROS) are elevated and affect the vessels by targeting several signalling cascades that are related to cell proliferation, differentiation and cell death (Ushio-Fukai *et al.*, 1998; Touyz *et al.*, 2001). In hypertension, their major sources are xanthine oxidize, uncoupled endothelial NO synthase and NAD(P)H oxidize which are activated by Ang II and the shear stress (Seshiah *et al.*, 2002; Landmesser *et al.*, 2003; Lassègue & Clempus, 2003). As mentioned before, the activation of redox-sensitive tyrosine kinases may mediate some of the vascular changes that occur in hypertension as ROS can mediate Ang II transactivation of EGFR. However, direct targets of ROS are protein tyrosine phosphatases (PTP) which together with tyrosine kinases control the level of phosphorylation in cells (Stoker, 2005). The redox regulated pathways are implicated in the arterial remodeling with the induction of the expression of pro-inflammatory molecules leading to the recruitment of inflammatory cells like IL-6 and to vascular inflammation (Simon *et al.*, 1998; Schieffer *et al.*, 2000). In addition, experimental studies *in vivo* and *in vitro* showed that under pro-hypertensive conditions endothelial cells and fibroblasts express elevated amounts of molecules adhesive for inflammatory cells being this expression mediated by ROS (Cheng *et al.*, 1998; Tummala *et al.*, 1999; Pueyo *et al.*, 2000).

The pathogenesis of arterial hypertension correlates with mitochondrial dysfunction, which includes mitochondrial energy deficiency leading to a mild respiratory uncoupling in vascular smooth cells and calcium overload (Postnov, 2001; Bernal-Mizrachi *et al.*, 2005). From studies in both patients and animal models of hypertension was observed an association between hypertension and mitochondrial uncoupling proteins (UCP). In physiological conditions, these mitochondrial anionic transporters regulate mitochondrial

membrane potential and ROS generation (Bernal-Mizrachi *et al.*, 2005). However, when the mitochondrial antioxidant defense system is impaired, ROS production is induced by the activity of monoamine oxidize by producing hydrogen peroxide, a major source of ROS (Youdim *et al.*, 2006) and the production of the protein p66Sh which binds to cytochrome c and acts as oxidoreductase catalyzing electron transfer from cytochrome c to oxygen, thus generating ROS (Giorgio *et al.*, 2005), (Booz & Baker, 1995; Berk *et al.*, 2007; Marín-García *et al.*, 2011) and leading to apoptosis, vascular wall remodeling and atherosclerosis (Napoli *et al.*, 2003).

VI. Diagnosis and Treatment recommendations according to the ESH/ESC Guidelines

The evaluation of hypertension involves blood pressure measurements, a medical history and physical examination and routine laboratory studies (Katakam *et al.*, 2008; Mancia *et al.*, 2013). These steps can help determine the presence of end-organ disease, the possible causes of hypertension, cardiovascular risk factors, baseline values for judge biochemical effects of therapy (Katakam *et al.*, 2008; Mancia *et al.*, 2013). Other studies may be obtained on the basis of clinical findings or in individuals with suspected secondary hypertension and/or evidence of target-organ disease, such as complete blood count, chest radiograph, 12-lead ECG, serum uric acid, serum creatinine (with estimation of GFR) and urine microalbumin (Mancia *et al.*, 2013).

In the 2003 and 2007 ESH/ESC guidelines a large number of randomized trials of antihypertensive therapy were reviewed. They concluded that the main benefits of antihypertensive treatment are due to lowering of BP per se and are largely independent of the drugs employed (Cifkova *et al.*, 2003; Mancia *et al.*, 2007; Mancia *et al.*, 2013).

In the Losartan Intervention For Endpoint Reduction in Hypertensives (LIFE) study, left ventricular hypertrophy regression was linearly related to the treatment induced BP changes (*the lower the better*) (Okin PM, 2003). In Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial (ONTARGET), the lowest BP achieved by the ramipril-telmisartan combination was associated with reduced proteinuria, but with a greater risk of acute renal failure and a similar cardiovascular risk (Liebson & Amsterdam,

2009). Several studies have shown that the regression of asymptomatic organ damage occurring during treatment reflects the treatment-induced reduction of morbid and fatal cardiovascular events (Mancia *et al.*, 2013).

Drug classes recommended for first line therapy are diuretics (including thiazides, chlorthalidone, and indapamide), β-adrenoceptor blockers, calcium channel blockers, angiotensin-converting enzyme (ACE) inhibitors, and angiotensin receptor blockers. All of them are suitable for the initiation and maintenance of antihypertensive therapy either as monotherapy or in some combinations (Mancia *et al.*, 2013). In the JNC8 guidelines, the diuretics were preferred for initial therapy over other classes, and in the British guidelines, excluded β-blockers from first line use except in people with angina or heart failure (Ritchie *et al.*, 2011; James *et al.*, 2014). Recent data suggesting that diuretics, such as, chlorthalidone and indapamide have better evidence for reduced cardiovascular events in AHT, than conventional thiazide diuretics was not supported (Roush *et al.*, 2012).

The 2013 ESH/ESC recommendations are based on consensus that resistant hypertensive patients should remove drugs that are shown not to lower BP; consider adding mineralocorticoid antagonist, amiloride, or doxazosin to the regimen; and consider renal denervation or baroreceptor stimulation if optimal drug therapy is ineffective (Mancia *et al.*, 2013). However these procedures are not available in most centers and are not used as routine clinical therapeutic strategies (Jennings & Touyz, 2013).

ESH/ESC guidelines recommend lifestyle modification for all patients with hypertension or prehypertension. Clinical studies show that the BP-lowering effects of targeted lifestyle modifications can be equivalent to drug monotherapy (Elmer *et al.*, 2006).

The recommended lifestyle modifications, that have been shown to be capable of reducing BP, are: reducing dietary sodium (5-6 g per day); cessation of smoking; moderation of alcohol consumption (to no more than 20–30 g of ethanol per day in men and to no more than 10–20 g of ethanol per day in women); increase the consumption of vegetables, fruits, and low-fat dairy products; weight reduction and maintenance (body-mass index up to 25 kg/m^2 and of waist circumference to <102 cm in men and <88 cm in

women); and regular physical exercise (at least 30 min of moderate dynamic exercise on 5 to 7 days per week) (Mancia *et al.*, 2013).

Patients with hypertension should be advised to eat fish at least twice a week and 300–400 g/day of fruit and vegetables. Soy milk appeared to lower BP when compared with skimmed cows' milk (Rivas *et al.*, 2002). With regard to coffee consumption, a recent systematic review found that most of the available studies were of insufficient quality to allow a firm recommendation to be given for or against coffee consumption as related to hypertension (Steffen *et al.*, 2012).

In relation to these lifestyles changes and effect in BP, it has been shown that reduction in sodium to about 5 g/day has a modest (1–2mmHg) SBP-lowering effect in normotensive individuals and a somewhat more pronounced effect (4–5mmHg) in hypertensive individuals (Dickinson *et al.*, 2006; Pimenta *et al.*, 2009; Graudal *et al.*, 2012). The Prevention And Treatment of Hypertension Study (PATHS) investigated the effects of alcohol reduction on BP and the intervention group had a 1.2/0.7mmHg greater reduction in BP than the control group at the end of the 6-month period (Cushman *et al.*, 1998). In a meta-analysis, the mean SBP and DBP reductions associated with an average weight loss of 5.1 kg were 4.4 and 3.6 mmHg, respectively (Neter *et al.*, 2003). Another meta-analysis of randomized controlled trials has shown that aerobic endurance training reduces resting SBP and DBP by 3.0/2.4mmHg overall and even by 6.9/ 4.9mmHg in hypertensive participants (Cornelissen & Fagard, 2005).

Alternative treatments such as vitamin C, coenzyme Q10, magnesium, and omega-3 fatty acids have been suggested for managing hypertension, but evidence for their effectiveness is lacking.

VII. Animal models of hypertension

The difficulty in studying a disease process such as hypertension begins with the fact that the aetiology of AHT is heterogeneous and involves complex interactions between genetically mechanisms and environmental factors (Takahashi & Smithies, 2004; Lerman *et al.*, 2005; Sarikonda *et al.*, 2009). Therefore, several experimental models have been developed to mimic the many facets of human AHT. The ideal animal model for AHT

research should have human-like cardiovascular anatomy, hemodynamics, and physiology; develop the human AHT characteristics and complications; allow studies in chronic stable AHT; and allow measurement of relevant hemodynamic and biochemical parameters (Doggrell & Brown, 1998; Lerman *et al.*, 2005). Inevitably, no species can consistently answer all of these needs, and experimental design and other constraints often dictate the choice of animal models for specific research applications.

There are several animal models of hypertension including renal (Goldblatt model), pharmacological or endocrine (deoxycorticosterone (DOCA)-salt rat) and genetic (SHR or Dahl salt sensitive rat) hypertension (Pinto *et al.*, 1998; Sun & Zhang, 2005) as shown in table 1.4. In this thesis, it was chosen the most common animal model of essential AHT, that is the spontaneously hypertensive rat (SHR), taking into account their characteristics and advantages over other models (Pinto *et al.*, 1998).

Table 1.4. Animal models of hypertension: renal, pharmacological or endocrine and genetic hypertension.

Animal model	Type of AHT	Description
Goldblatt	Renovascular hypertension	Clipping of the renal artery of one kidney with the other kidney removed (one kidney one clip) or retained (two kidney one clip). Can be carried out in different species including dog and rat.
DOCA-salt	Endocrine or pharmacological hypertension	Large doses of deoxycorticosterone (DOCA) along with salt and often removal of one kidney are used to induce hypertension
Ren2 over expression	Transgenic hypertension	Over expression of the mouse Ren2 gene in the rat causing an increase in renin and is related to severe hypertension
Sinoaortic denervation (SAD)	Neurological hypertension	SAD results in chronic hypertension in several species, including dog, cat, baboon and rabbit.
Dahl salt sensitive rats	Dietary/genetic hypertension	Genetic predisposition to develop severe hypertension following high salt intake
SHR	Genetic hypertension	Hypertension develops with age (see text for further details)
Stroke prone SHR	Genetic hypertension and cardiovascular disease	Related to the SHR, but with higher BP and predisposition for stroke (Nabika *et al.*, 2004)

The SHR strain was derived from the Wistar-Kyoto (WKY) rats and their WKY inbred non-hypertensive controls (Okamoto & Aoki, 1963). However it is important to focus that the SHR is not strictly an inbred strain, so there is still some genetic variability between breeding colonies from different establishments (Nabika *et al.*, 1991) and not all SHR necessarily develop hypertension. The SHR does not require surgical or pharmacological intervention to develop hypertension (unlike the Goldblatt or DOCA-salt models) (Zicha & Kunes, 1999) and is recognized as an excellent model of experimental AHT that can be used in clinical studies as a model of human essential AHT (Trippodo & Frohlich, 1981).

The SHR is born normotensive and systolic blood pressure (SBP) gradually increases from three weeks of age, becoming hypertensive by six weeks of age compared to age-matched normotensive controls (Dickhout & Lee, 1998). At 12 weeks of age, SBP is maintained at 180-200 mmHg in the SHR, whereas, the age-matched WKY has a SBP of 115-130 mmHg (Pinto *et al.*, 1998). According to Pinto *et al*., the SHRs older than 12 weeks of age reached a plateau phase (Pinto *et al.*, 1998). In the early stages of hypertension, SHR have an increased cardiac output, with normal total peripheral resistance (TPR). Since the SHR progresses into the established hypertension state, the cardiac output returns to normal values and the hypertrophied blood vessels induce a TPR increase (Smith & Hutchins, 1979). The SHR also displayed increased heart rate (HR) from two weeks old, reduced baroreflex sensitivity and increased chemoreflex sensitivity (Przybylski, 1981; Hayward *et al.*, 1999). With the progress of hypertension, the SHR progressively develops (between 6 and 24 months of age) structural alterations in the heart, which are associated with progressive cardiac hypertrophy (Engelmann *et al.*, 1987). As this is not a strictly inbred strain, individual variations in the genetic background of both SHR and particularly of their control strain may significantly influence the resulting end-organ changes (Pinto *et al.*, 1998).

This animal model has the advantage of being commercially available and has been extensively studied, so it is physiologically well characterized. Moreover, it is a chronic stable model, producing symptoms which are predictable and controllable (Doggrell & Brown, 1998). For presenting a relative short life (the normal life spans of SHR is 1.5–2.5 years vs 2.5–3 years of WKY), being small, have relatively low cost and easy maintenance

in animal houses, the SHR are frequently used to study the genetic determinants and the pathophysiological changes in the essential hypertension (Folkow & Svanborg, 1993).

Another advantage of the SHR is that it follows the same progression of hypertension as human hypertension with pre-hypertensive, developing and sustained hypertensive phases, with each phase lasting at least several weeks (Folkow, 1993). However the SHR differs from human hypertension in that SHR reproducibly develop hypertension in young adulthood rather than in middle age as in humans (Doggrell & Brown, 1998). The SHR also have other similarities to human essential hypertension: the AHT is multi-factorial, involving a neurogenic component (increased sympathetic nerve activity and total peripheral resistance) and responds to anti-hypertensive drugs prescribed to human patients (Pinto *et al.*, 1998). As in humans, hypertension develops more rapidly and becomes more severe in male than female SHR (Iams *et al.*, 1979; Maris *et al.*, 2005). So the male SHR is more commonly used as a model of established human hypertension (Doggrell & Brown, 1998).

Moreover, the slow onset of disease in SHR allows the investigation of these animals before the onset of hypertension. This allows researchers to differentiate between changes that are the cause and those that are secondary to the onset of hypertension. It also allows the study of therapeutic agents to prevent the development of hypertension in the SHR, as well, as investigations of possible drugs that can minimize or reverse established hypertension in adult SHR.

The common criticism of the SHR model is the doubt about the cause of the onset of the disease. However, the genetic mechanisms of hypertension in SHR have been frequently attributed to both neural and vascular alterations observed in these animals (Lerman *et al.*, 2005).

1.2 PATHOPHYSIOLOGY OF NEUROGENIC HYPERTENSION

I. Hypertension and Sympathetic Nervous System

I a. "Neurogenic" Essential Hypertension: Historical Antecedents

The historical antecedents help us to understanding the importance of sympathetic nervous system (SNS) pathophysiology in the pathogenesis of essential hypertension: 1st) the anatomical description of sympathetic nerves and ganglia and their identification as pressor nerves; 2nd) surgical sympathectomy as an antihypertensive therapy; 3rd) identification of noradrenaline as a sympathetic transmitter; 4th) development of anti-adrenergic antipertensive drugs; 5th) techniques developed for measuring human sympathetic activity and 6th) SNS activation demonstrated in AHT (Parati & Esler, 2012).

Thomas Willis discovered the workings of the sympathetic nervous system and presented the findings in “The Anatomy of the Brain and Nerves” (Zimmer, 2004). They identified these as blood pressure-raising "pressor nerves", suggesting, for the first time, that they might cause AHT.

In the early decades of the twentieth century, with the high mortality of severe AHT and with no effective pharmacological treatment, a number of operations on the sympathetic nervous system were devised in order to lower BP. The radical lumbodorsal splanchnicectomy, developed in 1938 by Smithwick and co-workers (Smithwick *et al.*, 1956), which incorporated surgical section of accessible nerves in the thorax and abdomen and transaction and clipping of the sympathetic chain, lowered the BP and reduced the mortality, but at the cost of often incapacitating side effects, particularly disabling postural hypotension.

By the late 1960s, it had been demonstrated that in the developmental phase of essential hypertension, cardiac output and heart rate were commonly elevated, intimating the presence of probable sympathetic nervous system activation (Julius & Conway, 1968). Another study demonstrated that this increase of cardiac output in the early phases of essential hypertension is gradually converted, over the course of many years, to the hemodynamic pattern of normal cardiac output and high vascular resistance, considered typical of the hypertensive condition (Lund-Johansen, 1989).

Therefore the idea of neurogenically mediated hypertension has emerged. So the majority of antihypertensive drugs entering clinical practice that antagonized the autonomic nervous system were ganglionic blockers or drugs that specifically antagonized its sympathetic division, like central sympathetic inhibitors, such as methyldopa and clonidine, sympathetic neuronal blockers, such as guanethidine, and alpha- and beta-adrenergic blockers (Parati & Esler, 2012). The effectiveness of these drugs showed that the SNS was important in the pathogenesis of essential hypertension.

The antihypertensive drugs used most widely, ranking highest in international treatment guidelines, include the angiotensin receptor blockers, angiotensin-converting enzyme inhibitors, calcium channel blockers, and diuretics. Antiadrenergic drugs are not prominent in the guidelines, because the currently available antiadrenergic drugs produce frequent metabolic and other adverse effects and are less effective, since they tend to reduce rates of myocardial infarction and stroke less than the first-line drugs.

I b. Activation of the Sympathetic Nervous System in Essential Hypertension

The role of the SNS in the pathogenesis of AHT only became clear in the last few years. Until a few decades ago it was thought that the SNS acted mainly in the control of BP in the short term, having almost no influence on their long term control, being exercised primarily by control of salt and water balance, through the renin-angiotensin-aldosterone system.

However, in the last 20 years due to the refinement of the assessment of sympathetic cardiovascular function, the concepts regarding the role of the SNS in the regulation of BP were drastically altered (Mark, 1996).

At present, it is known that overactivity of the SNS contributes to the onset, development and maintenance of hypertension (Guyenet, 2006; Tsioufis *et al.*, 2011). In fact, studies in humans and animals clearly demonstrate an increased sympathetic nerve activity in the hypertensive state (Grassi, 2004b; Guyenet, 2006; Fisher & Paton, 2012). The same was not observed in subjects with secondary hypertension (Grassi *et al.*, 1998; Grassi, 2004a, 2009).

The increase of sympathetic outflow to the heart results in increased cardiac output and neurally mediated vasoconstriction leading to elevated blood pressure values (Schlaich *et al.*, 2012). Excessive sympathetic activity may also contribute to vascular smooth and cardiac muscle hypertrophy, organ hypoperfusion and inflammation (Zubcevic *et al.*, 2011).

In white coat and borderline hypertensive patients, the sympathetic nerve activity to the arterioles supplying skeletal muscle is already raised compared to healthy individuals (Grassi, 2004a; Smith *et al.*, 2004). Also in normotensive subjects with family history of AHT the activity of the SNS is increased (Yamada *et al.*, 1988). These data seem to suggest that the excitation of the sympathetic nervous system precedes the onset of hypertension and that may be the cause of this condition.

Furthermore, it is established, after several studies, that the activity of the SNS increases progressively and in parallel with the stages of AHT (Smith *et al.*, 2004; Tsioufis *et al.*, 2011).

The sympathetic fibres' recording from the renal plexus and the measurement of the spillover of norepinephrine demonstrated that the efferent SNS to the kidneys, heart and vasculature of skeletal muscle duplicate in individuals with essential AHT compared to normotensive individuals (Esler *et al.*, 1988; Grassi *et al.*, 1998; Petersson *et al.*, 2002; Schlaich *et al.*, 2004; Lambert *et al.*, 2007).

Studies in obese patients showed that there is renal sympathetic activation with a minimal involvement of sympathetic outflow to the heart. In fact, in many obese hypertensive patients cardiac norepinephrine is reduced (Rumantir *et al.*, 1999). On the other hand, in normal-weight patients with hypertension, both cardiac and renal sympathetic outflows are activated (Esler *et al.*, 1985; Esler *et al.*, 1988; Rumantir *et al.*, 1999).

Single-fibre sympathetic recording demonstrates increased CNS sympathetic outflow, with increased fibre-firing frequencies and multiple firings within a cardiac cycle, not seen in health conditions (Greenwood *et al.*, 1999; Lambert *et al.*, 2007).

It is estimated that the AHT with neurogenic cause is no less than 50% of all cases of essential AHT. This is based the proportion of untreated patients with essential

hypertension who have demonstrable sympathetic excitation, and in the number in whom substantial BP lowering is achieved, as well as the extent of this lowering with anti-adrenergic drugs (Parati & Esler, 2012).

Recent evidence also suggests that afferent sensory nerves from the kidneys that project to the brain are an important source of sympathetic activation. In fact, in patients with resistant hypertension that respond inadequately to concurrent treatment with multiple antihypertensive drug classes, ablation of the renal sympathetic nerves with an endovascular radiofrequency technique lowered blood pressure remarkably (Krum *et al.*, 2009; Esler *et al.*, 2010).

Activation of the renal sympathetic nerves has been seen to be pivotal. The introduction of radiofrequency renal sympathetic nerve ablation as an effective treatment for patients with essential hypertension now adds compelling empirical evidence (Krum *et al.*, 2009; Esler *et al.*, 2010).

So, the reduction of the enhanced sympathetic activity has been considered as an antihypertensive strategy (Del Colle *et al.*, 2007; Biaggioni, 2008; Signolet *et al.*, 2008; Fisher & Fadel, 2010; Grassi *et al.*, 2010). However, the mechanism of sympathetic activation in AHT is not well known.

The specific causes of the increased sympathetic activity in essential AHT remain enigmatic, although it is known that the interaction of genetic influences with behavioural and lifestyle factors are important.

Genetic influence on sympathetic activity in essential AHT may be polygenetic and thus more difficult to identify, perhaps involving interactions of overweight, mental stress and dietary sodium intake with the SNS (Chandola *et al.*, 2006). However, there is evidence that essential AHT is approximately 30%-40% heritable (Longini *et al.*, 1984).

Physical inactivity also appears to be important in AHT. In fact the observation that aerobic exercise training in sedentary people reduces sympathetic nervous activity and preferentially renal sympathetic outflow supports this concept (Somers *et al.*, 1995).

Recent experience with radiofrequency renal nerve ablation in patients with resistant hypertension, in which the denervation procedure reduces whole-body sympathetic activity, indicates that a "renal injury" signal from the kidneys to the brain must exist also

in these patients, contributing to the chronic sympathetic activation evident in these patients (Shibao *et al.*, 2007; Schlaich *et al.*, 2009).

Adipokines, O2 and CO2 blood concentration, endothelial factors, aldosterone, angiotensin II, insulin resistance and baroreflex impairment, have also been implicated in sympathoexcitation.

The hypothesis, which is the basis of this thesis, is that the hyperactivity of the SNS can be caused by inappropriate increase in the activity of the brain centres. Hence the importance of whether the silencing and/or decreased activity of central nuclei that coordinate sympathetic activity and that are potentially deregulated in AHT may be sufficient to cause changes at the peripheral level.

In experiments that were initially designed to produce analgesia in patients with chronic neuropathic pain, Green et al. (2006) demonstrated that electrical stimulation of midbrain structures produces depressor responses. Whether these would be both powerful enough to off- set the high blood pressure in a hypertensive patient and persist chronically for effective treatment remains to be determined (Green *et al.*, 2006).

II. Hypertension and Central Nervous System

The Central Nervous System (CNS) plays an important role in the short term control of BP, but its contribution to the chronic control of BP is not yet clear. In fact, research in the past years has been directed to essential hypertension with possible neurogenic cause.

As mentioned above, several studies suggest that the SNS is a predominant factor for the development, maintenance and progression of essential hypertension. The SNS hyperactivity found in individuals with essential hypertension can be caused by inappropriate increase in the activity of sympathetic-excitatory regions of the CNS. The most mentioned sympathetic excitatory regions are the Paraventricular Nucleus of the Hypothalamus (PVN) and the Rostral Ventrolateral Medulla (RVLM), which will be described below.

II a. Paraventricular nucleus of the hypothalamus or PVN

The paraventricular nucleus (PVN) is a major sympatoexcitatory area, that becomes more active under conditions of hypertension such as in the spontaneously hypertensive rat (SHR) model (Allen, 2002). Some authors have referred to this region as a command nucleus providing feed forward excitatory synaptic drives to coordinate lower brainstem cardiovascular and respiratory motor activity and where there is an integration of autonomic and neuroendocrine responses (Swanson & Sawchenko, 1980, 1983; Dampney *et al.*, 2005).

The PVN is also a critical component in the pathways that control the blood volume, since this nucleus receives afferent information from receptors located in the right atrium and in the inferior vena cava, which are sensitive to small (8-10%) volume changes (Lovick & Coote, 1988) van Giersbergen, 1992).

The PVN is located in the forebrain and can be divided into several distinct subnuclei (Benarroch, 2005). In the rat, the PVN has approximately 21500 neurones arranged into eight subnuclei (Swanson & Kuypers, 1980; Kiss *et al.*, 1991). It contains two different types of neurones: the magnocellular and parvocellular. The magnocellular neurons (arranged into three subnuclei) are large cells that project to the posterior pituitary where they synthesize and release oxytocin (OT) and vasopressin (VP) into the circulation (Armstrong & Hatton, 1980; Sawchenko & Swanson, 1982; Kiss *et al.*, 1991; Benarroch, 2005).

VP acts upon the kidney to promote antidiuresis and upon the blood vessels to cause vasoconstriction. In addition, baroreceptor unloading stimulates VP release. OT is best known in association with reproductive functions in the female animal, such as parturition and milk ejection, although it is co-secreted with VP in response to osmotic and blood volume challenges (Hatton, 1990). It also contract blood vessels when present in relatively larger amounts and hence can cause increases in BP (Petty, 1987; Richard *et al.*, 1991).

Plasma OT have been shown to increase kidney sodium excretion in the conscious rats (Verbalis *et al.*, 1991). OT acts as a neuromodulator within the brainstem; when microinjected into the NTS, OT caused hypertension and tachycardia (Vela *et al.*, 2010).

In addition, OT knockout mice were slightly hypotensive suggesting that OT plays a role in tonic MBP maintenance (Michelini *et al.*, 2003).

Smaller neurones form the parvocellular division of the PVN and can be divided into five subnuclei (Swanson & Kuypers, 1980). It is from amongst these neurones that projections arise to innervate different groups of autonomic neurones in the brainstem and spinal cord (Fig.1-6).

The projections in the PVN have been identified using retrograde and anti-retrograde markers microinjected into the PVN, rostral ventrolateral medulla (RVLM) and/or in the intermediolateral (IML) cell column of the spinal cord (Ranson *et al.*, 1998; Shafton *et al.*, 1998; Motawei *et al.*, 1999; Pyner & Coote, 1999, 2000; Pyner *et al.*, 2001). In the end, these projections synapse on their effector organs - the heart, blood vessels and kidneys.

The PVN project to regions such as the nucleus tractus solitari (NTS), dorsal motor nucleus of the vagus (DMNV), periaqueductal gray (PAG), parabrachial nucleus (PBN), RVLM, caudal ventrolateral medulla (CVLM) and intermediolateral (IML) cell column (Fig. 1-6) (Swanson & Kuypers, 1980; Luiten *et al.*, 1985; Holstege, 1987; Michelini & Morris, 1999; Pyner, 2009).

The parvocellular cells secrete vasopressin and corticotropin-releasing hormone (or corticotrophin releasing factor, CRF) (Engelmann *et al.*, 2004). These hormones are released in pulses into the hypophyseal portal system by the parvocellular neurones approximately every 30 minutes (Engelmann *et al.*, 2004; Benarroch, 2005). The release of CRF also promotes the release of adrenocortico tropic hormone (ACTH) from the adenohypophysis (Sawchenko, 1987b, a).

The parvocellular neurones seem to form three separate projections that modulate the activity of the SNS (Pyner, 2009). There are direct projections from the PVN to the RVLM and from the PVN to the IML and an indirect projection, which goes to RVLM and the IML, both critical regions in the control of the SNS (Dampney, 1994; Badoer *et al.*, 1997; Badoer, 2001).

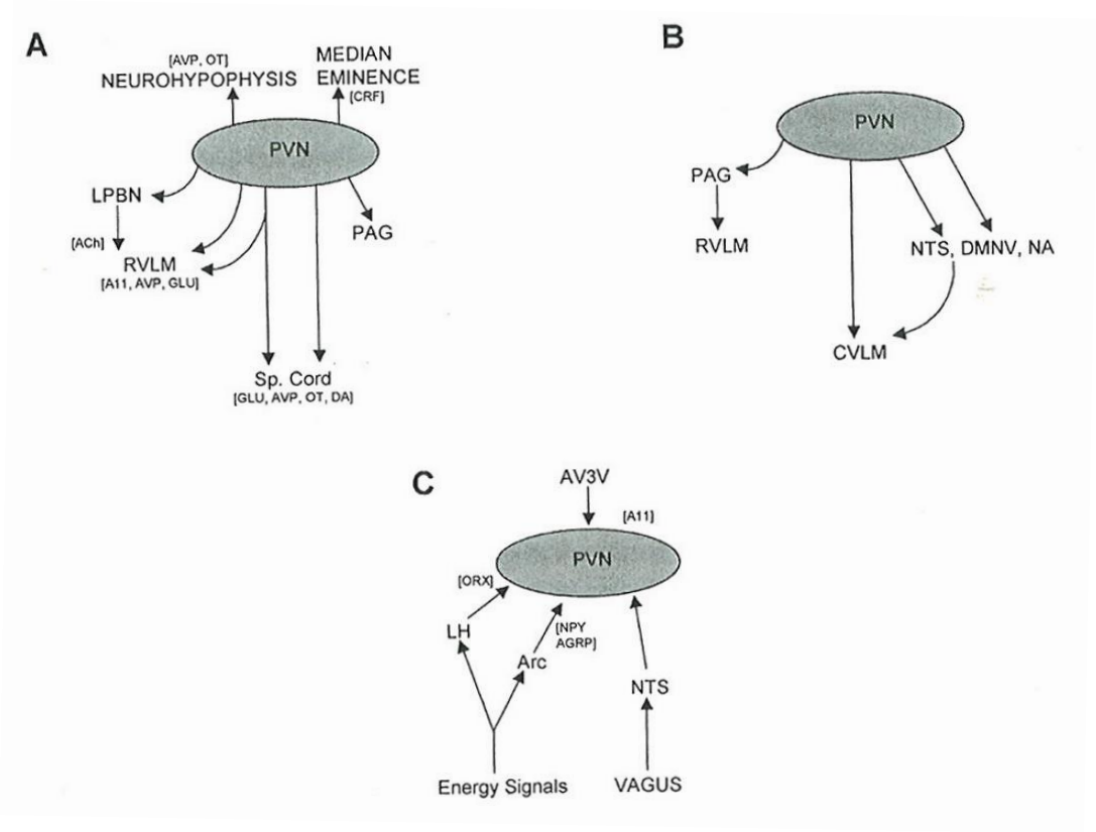


Fig. 1-6. Diagram illustrating the principal autonomic efferent projections from the PVN (A, B) and the autonomic afferent inputs to the PVN (C). PVN, paraventricular nucleus; LH, lateral hypothalamus; Arc, arcuate nucleus; AV3V, anteroventrolateral region of 3rd ventricle; LPBN, lateral parabrachial nucleus; RVLM, rostral ventrolateral medulla; CVLM, caudal ventrolateral medulla; Sp Cord, , thoraco-lumbar spinal cord; PAG, periaqueductal grey; NTS, nucleus tractus solitari; DMNV, dorsal motor nucleus of the vagus; NA, nucleus ambiguous; VP, vasopressin; CRF, corticotrophin releasing factor; Ang II, Angiotensin II; GLU, glutamate; OT, oxytocin; DA, dopamine; ORX, orexin; NPY, neuropeptide Y; AGRP, agouti related protein. Adapted from John Coote, Neural mechanisms of cardiovascular regulation, 2004.

Hence the PVN is considered one of the five major groups of sympathetic premotor neuronal cells (Strack *et al.*, 1989; Dampney, 1994) and due to its connections with RVLM also influences the vasomotor sympathetic nerve discharge (Shafton *et al.*, 1998; Yang & Coote, 1998).

In fact the PVN appears to be involved in the regulation of sympathetic activity (Coote, 2005). For example, electrolytic lesions of the PVN in SHR causes a decrease in BP, and this decrease was associated with reduced SNS activity which occurred without changes in the secretion of VP (Takeda *et al.*, 1991).

In addition, microinjection of muscimol in the PVN in SHR and in Dahl salt-sensitive rats resulted in a decrease in MBP and caused a reduction in the SNS activity without affecting the MBP of their control animals (Allen, 2002; Ito *et al.*, 2003). However, there are conflicting results regarding the BP observed in conscious and anesthetized rats (Badoer *et al.*, 2002; Li *et al.*, 2007). Other studies show that GABAergic synaptic inputs to the PVN inhibit the sympathetic tone and BP (Decavel & Van den Pol, 1990; Zhang & Patel, 1998; Zhang *et al.*, 2002). In contrast, PVN microinjection of bicuculline (a $GABA_A$ receptor antagonist) or glutamate elevated sympathetic nerve activity causing hypertension in anesthetized and conscious rats (Kannan *et al.*, 1989; Zhang *et al.*, 2002).

PVN lesions or the transection of the brain caudal to the hypothalamus promotes a decrease in blood pressure in SHR but not in WKY rats (Yamori & Okamoto, 1969; Goto et al., 1981; Ciriello et al., 1984; Herzig et al., 1991; Takeda et al., 1991).

In the ***PVN-spinal sympathetic pathway*** there is now convincing evidence that VP and OT act as neurotransmitters, since both peptides depolarise sympathetic preganglionic neurones recorded in vitro in slices of spinal cord (Ma & Dun, 1985; Sermasi & Coote, 1994; Desaulles *et al.*, 1995; Kolaj & Renaud, 1998). Also, VP or OT applied iontophoretically alter the firing rate of cardiovascular-like sympathetic preganglionic neurones (Gilbey *et al.*, 1982; Backman & Henry, 1984). Furthermore, VP or OT given intrathecally to the thoracic cord increase activity in renal sympathetic nerves (Porter & Brody, 1986; Tan & Tsou, 1986; Riphagen & Pittman, 1989a; Malpas & Coote, 1994; Yang *et al.*, 2002). It has also been shown that pressor responses or increases in renal sympathetic nerve activity elicited by PVN stimulation can be selectively blocked by intrathecally applied V1a antagonist (Riphagen & Pittman, 1989b; Malpas & Coote, 1994; Yang *et al.*, 2002). However, it was not possible to show the PVN-OT dependent effect on renal sympathetic outflow (Yang *et al.*, 2002). One hypothesis is that the OT pathway terminates on different target specified sympathetic neurones compared to the VP pathway. In fact, there also appears to be a difference in the sensitivity of sympathetic neurones to the peptides: some neurones in the upper thoracic segments display selective action to OT, whereas on neurones in the lower thoracic segments wich are especially sensitive to VP (Sermasi & Coote, 1994; Desaulles *et al.*, 1995).

The ***PVN-NTS pathway*** is involved in homeostasis and responses to stressful situations (Engelmann *et al.*, 2004). Stimulation of this pathway causes the synthesis and release of VP and OT into the blood stream (Loewy & Spyer, 1990b). There is also persuasive evidence that part of the PVN-NTS projection inhibits arterial baroreceptor reflex transmission, since microinjection of the VP or OT peptides causes an increase in BP and HR (Matsuguchi *et al.*, 1982). Also, lesions of the PVN increase a baroreceptor-induced inhibition of lumbar sympathetic nerve activity (Darlington *et al.*, 1988; Patel & Schmid, 1988).

Relative to the ***PVN-RVLM pathway*** it has been shown that the activation of PVN neurones induce sympathoexcitatory and pressor responses via excitatory connections with RVLM (Coote *et al.*, 1998; Yang & Coote, 1998). It also appears that glutamate (GLU) and VP neurons, as well as other PVN neuronal phenotypes are important in the RVLM (Gómez *et al.*, 1993; Yang *et al.*, 2001). Terminals of PVN neurones containing CRF have been demonstrated in the RVLM region and bilateral microinjection of CRF into this region increases BP (Milner *et al.*, 1993). There is also evidence that the PVN can excite neurones in the RVLM by activation of angiotensin receptors, but is not clear how direct this pathway is (Tagawa & Dampney, 1999; Tagawa *et al.*, 2000).

The PVN also receives projections from the caudal ventrolateral medulla (CVLM) - the ***PVN-CVLM pathway*** - and the neurons in this area also receive inputs from neurones in the NTS (Kawano & Masuko, 1996). This suggests that the NTS has a direct and an indirect (which includes CVLM) projection to the PVN (Kawano & Masuko, 1996). In a study that experimentally induced hypotension resulted in c-fos expression in the PVN neurones that project to the NTS and to the CVLM, indicating that these connections are reciprocal (Krukoff *et al.*, 1997).

Similarly, distinct descending projections to the dorsal motor vagus (DMV), the nucleus ambiguous (NA) and the NTS provide the anatomical substrate for PVN influences on parasympathetic activity and baroreflex control, respectively (Armstrong *et al.*, 1980; Swanson & Kuypers, 1980; Ranson *et al.*, 1998).

It is well established that the parvocellular neurones in the PVN influence food intake and energy expenditure (Woods & D'Alessio, 2008) and these same neurones also express

receptors of Ang II type1 (AT1) (Lenkei *et al.*, 1997). It is known that these receptors play an important role in AHT, however, the central mechanisms by which these receptors contribute to sympathetic-excitation remain unclear.

Results of one study indicated that the increased activity of AT1 receptors in the PVN contributes to the increase of afferent sympathetic reflex and the cardiac sympathetic-excitation in rats with renovascular hypertension (Chen *et al.*, 2011). Another study shows that blockade of AT1 receptors in the PVN reduces renal sympathetic excitation induced by central hyperosmolality (Chen & Toney, 2001).

Thus, through modulatory actions on the baroreceptor reflex, sympathetic and parasympathetic nerve activity, the PVN is in a pivotal position to contribute to the short and long-term control of the cardiovascular system.

II b. Rostral Ventrolateral Medulla or RVLM

The RVLM lies ventral to the rostral part of the nucleus ambiguous (NA) and to the Bötzinger complex and caudal to the facial nucleus (Dampney, 1994; Janig, 2006b).

The RVLM neurons project to the sympathetic preganglionic neurones in the intermediolateral (IML) cell column of the spinal cord (Guertzenstein & Silver, 1974; Dampney, 1994; Leman *et al.*, 2000; Card *et al.*, 2006b) and receive a direct glutamatergic projection from the NTS, believed to be part of the peripheral chemoreflex (Ross *et al.*, 1985; Koshiya & Guyenet, 1996b; Nosjean *et al.*, 1998). There are also projections from the PVN to the RVLM (Kantzides & Badoer, 2005; Pyner, 2009) (see IIa).

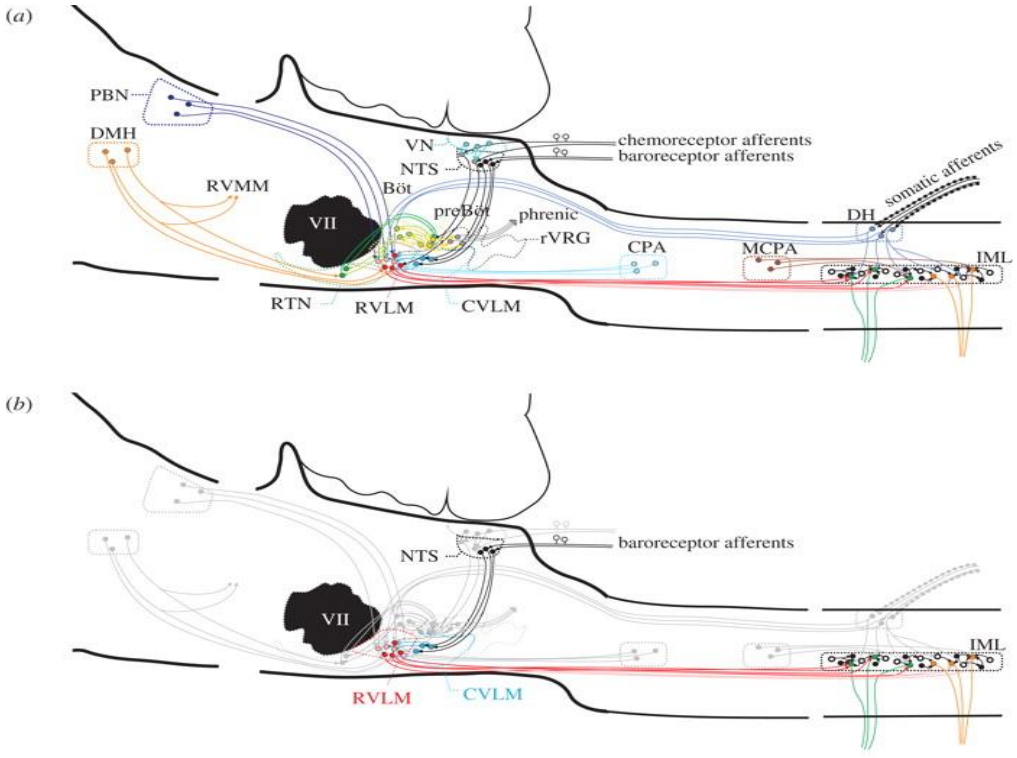
The RVLM plays an important role in BP control. In fact it has been shown that electrical or chemical stimulation (with glutamate) causes an increase in BP and HR in the cat (Guertzenstein & Silver, 1974) and in the rat (Ross *et al.*, 1984). On the other hand, inhibition of RVLM in the conscious rat causes a chronic decrease in BP, HR and in the SNS activity (Dampney, 1994; Kishi *et al.*, 2001).

Previous studies suggested that in SHRs there is an increased excitatory drive from RVLM neurons that is associated with an elevated sympathetic outflow (Bergamaschi *et al.*, 1995; Ito *et al.*, 2000, 2001; Ito *et al.*, 2002; Ito *et al.*, 2003). It was also shown that the

RVLM of SHR has a higher density of AT1 receptors (Hu *et al.*, 2002) and the blockade of these receptors in this area causes an increase in baroreflex sensitivity (Gao *et al.*, 2004) and a reduction in BP (Ito *et al.*, 2002) in SHR rats but not in WKY controls.

The RVLM of rats shows some viscerotopic organization according to the type of sympathetic preganglionic neurons it projects to (Beluli & Weaver, 1991; Dampney, 1994; McAllen & May, 1994). Sympathetic renal vasoconstrictors premotor neurones are located in the rostral area of the RVLM and the sympathetic visceral vasoconstrictive premotor neurones are more caudal (Janig, 2006a). This RVLM connection with the sympathetic preganglionic neurones is vital in maintaining the basal BP. In anesthetized animals, acute bilateral lesions of the RVLM result in a decrease in BP (Guertzenstein & Silver, 1974; Dampney & Moon, 1980). Little is known about the role of RVLM after chronic lesions in conscious rats.

Figure 1-7 illustrates the neurons responsible for maintaining BP. There is a small nucleus in the rostral part of the RVLM in rats that are important, since any intervention that decreases or eliminates its normal function causes an acute decrease in sympathetic activity, all sympathetic reflex activity is eliminated and BP decreases to a level similar to that seen after high spinal cord transection (Schreihofer et al., 2005; Braga et al., 2007).



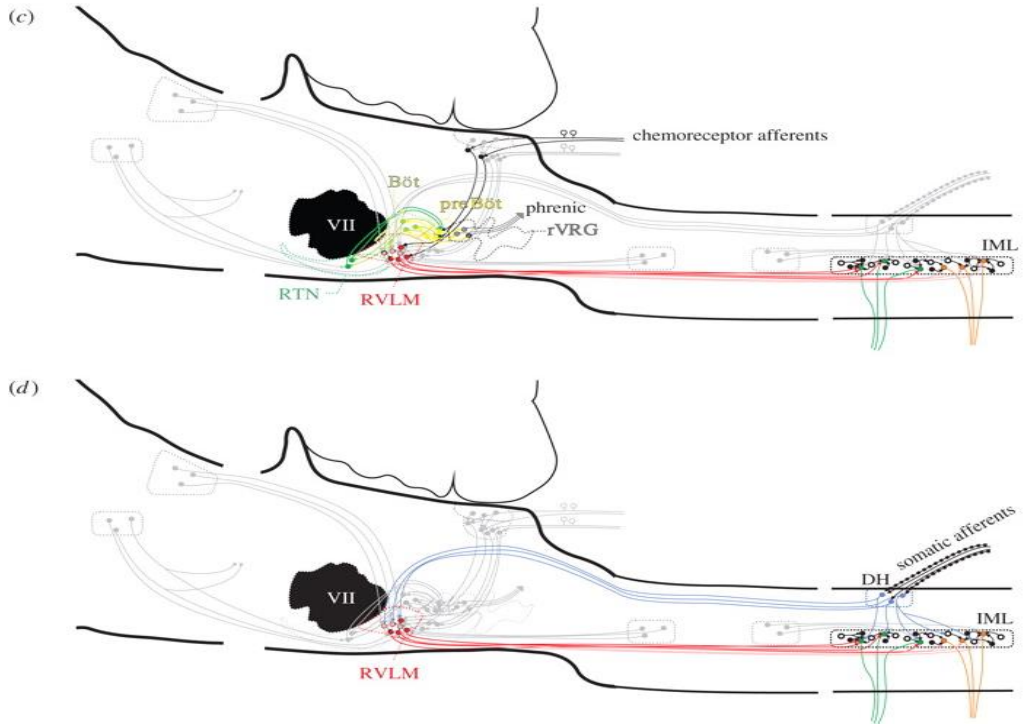


Figure 1-7. A diagram of pathways in the regulation of the cardiorespiratory system. (a) all pathways overlapped. The bulbospinal red pathways are in the RVLM and integrate information from the centre and the periphery. The output from this nucleus is crucial for maintaining normal sympathetic tone. PBN, parabrachial nucleus; DMH, dorsomedial hypothalamus; CVLM, caudal ventrolateral medulla; VLM, ventrolateral medulla; rVRG, rostral ventral respiratory group; CPA, caudal pressor area; MCPA, medullo cervical pressor area; IML, intermediolateral cell column; RVMM, rostral ventromedial medulla; VII, facial nucleus; RTN, retrotrapezoid nucleus; preBöt, preBötzinger neurons; VN, vestibular nucleus. (b) The baroreflex pathway is shown on its own. Stretch receptor afferent neurons from the aortic arch and carotid sinus and the neurons synapse in the nucleus tractus solitarius (NTS). Neurons in the NTS then activate inhibitory neurons (blue) in the caudal ventrolateral medulla, which in turn inhibit the neurons in the RVLM; this intense gamma-aminobutyric acid (GABA)-mediated inhibition inhibits sympathetic outflow, causing blood pressure and sympathetic nerve activity to fall. Note also the yellow respiratory neurons that modulate the activity of the cardiovascular neurons (also in c). (c) The pathways for peripheral and central chemoreceptors are shown. Peripheral chemoreception emanates from the carotid body. Neurons terminate in the medial NTS (like the baroreceptors). From here, the excitatory information passes to both respiratory and cardiovascular neurons. (d) The somatosympathetic pathway is shown in an abbreviated form. Afferent nociceptive pathways enter the spinal cord in the dorsal roots, activate circuits locally, and at several stations throughout the neuraxis including the RVLM. This pathway is excitatory and results in the appearance of a variable number of peaks in sympathetic nerve activity, depending on which nerve is recorded from. In the case of the greater splanchnic nerve, this is generally two peaks. Adapted from Pilowsky PM, *Differential regulation of the central neural cardiorespiratory system by metabotropic neurotransmitters*, 2009.

About 50-70% of the neurons of RVLM are part of the C1 catecholaminergic group (Sved *et al.*, 1994; Schreihofer & Guyenet, 1997; Madden & Sved, 2003a). The C1 cells are excitatory neurons whose ongoing discharges are essential to maintain resting sympathetic tone and BP.

These neurons are highly active at rest and powerfully inhibited by activation of arterial baroreceptors. However, it should be noted that both C1 and non C1-catecholaminergic neurones expressed mouse C-fos in response to hypotension, suggesting that both cell types are barosensitive (Chan & Sawchenko, 1994; Sved *et al.*, 1994).

Furthermore, in most instances, the C1 cells display a discharge pattern that is highly correlated with that of the sympathetic efferents that innervate the heart, kidney or blood vessels of the skeletal muscles and splanchnic area (Sun & Reis, 1996).

Initially, it was believed that C1 neurons were responsible for regulating sympathetic vasomotor pathways through the release of adrenaline in the spinal cord (Goodchild *et al.*, 1984; Ross *et al.*, 1984), but it became clear that both C1 and non-C1 neurons also release glutamate (Phillips *et al.*, 2001; Stornetta *et al.*, 2002; Morrison, 2003).

Glutamate is expressed in most of the barosensitive sympathetic preganglionic neurones that project to RVLM. The release of glutamate agonists as well as electrical stimulation of the RVLM caused excitation of sympathetic preganglionic neurons and this excitation could be blocked with glutamate antagonists (Morrison, 2003).

The actual role of adrenaline released at the level of sympathetic preganglionic neurons still remains unclear (Bolme *et al.*, 1974). The possibility is that it exerts complex effects depending on the post-synaptic receptor present and if an inhibitory interneuron is interposed (Shi *et al.*, 1988; Coote & Lewis, 1995).

The specific contribution of bulbospinal C1 cells to the generation of sympathetic vasomotor tone was showed in the anesthetized rat by a selective destruction (using an anti-DßH antibody) of over 80% of the C1 cells. It promoted a slight but significant (~ 10 mmHg) BP reduction and also attenuated the sympathetic tonus involved in baroreflex (determined by reflex change in HR in response to a decrease in mean BP) with little effect on the parasympathetic tonus (Guyenet *et al.*, 2001; Madden & Sved, 2003a, b).

The C1 cells project into central regions involved in cardiovascular control, including the PVN and the ventrolateral column of the PAG (Guyenet *et al.*, 2001; Card *et al.*, 2006a). Furthermore, some of the C1 cells of the RVLM project into chromaffin cells which control the release of adrenaline, whereas the barosensitive non-C1 RVLM cells control the release of noradrenaline by chromaffin cells (Morrison & Cao, 2000).

The RVLM displays a tonic activity in sympathetic preganglionic neurones. The *"pacemaker hypothesis"* proposed by Guyenet was based on studies using intracellular recording of the RVLM in medullary slices. However, the source of this pacemaker activity is unknown. There are two hypotheses: 1) there are pacemaker neurones within the RVLM or 2) the tonic input comes from a network of other brain regions (Guyenet, 2006).

The pacemaker properties are voltage dependent and not dependent on synaptic input. The pacemaker properties were attributed to the presence of a persistent sodium current (Kangrga & Loewy, 1995).

The pacemaker theory is based on the observation made in brain slices of the RVLM area, where there were some spontaneously active cells (Sun *et al.*, 1988a) that did not belong to C1 group (Sun *et al.*, 1988b). On the other hand, other authors reported technical difficulties when trying to identify pacemaker and non-pacemaker C1 cells within the RVLM (Kangrga & Loewy, 1995). However, in vivo, it has not been possible to identify spontaneously active pacemaker cells in the RVLM (Lipski *et al.*, 1996; Sved *et al.*, 2001).

So, based mainly on anaesthetized animals, the RVLM has been shown to be a pivotal area regulating cardiovascular sympathetic tone. Therefore, the increased activity of RVLM neurons is suspected to contribute to the increased sympathetic tone associated with AHT.

II c. Medulla Cervical Pressure Area or MCPA

The medullo-cervical pressor area (MCPA) is recently discovered sympathoexcitatory region that is located in the most ventrolateral medulla that extends caudally from the medulla at the level of the caudal pole of the inferior olive to the fourth cervical segment and contains spinally projecting neurons (which are neurochemically heterogeneous) that directly innervate the sympathetic preganglionic neurons (Seyedabadi *et al.*, 2006).

This pressor area is distinct from the caudal pressor area (CPA), because it is not dependent on the integrity of the RVLM and does not appear to mediate its effects via suprabulbar regions but via bulbospinal sympathetic neurons in the region.

Using retrograde tracing it was showed that MCPA neurons project to thoracic levels (which are neurochemically heterogeneous) that directly innervate the sympathetic preganglionic neurons (Seyedabadi *et al.*, 2006). On the other hand, another study suggested the possibility of a projection from the MCPA to the RVLM, since chemical stimulation in a region in the very caudal medulla activated bulbospinal barosensitive neurons of the RVLM (Campos & McAllen, 1999).

Other studies have demonstrated that bilateral RVLM blockade eliminates the responsiveness of the more rostrally located CPA (Gordon & McCann, 1988; Possas *et al.*, 1994; Natarajan & Morrison, 2000). In contrast, responses evoked from the MCPA are unaffected by bilateral RVLM blockade (Seyedabadi *et al.*, 2006). Thus, it seems that the MCPA does not appear to play a role in maintaining vasomotor tone after RVLM blockade and is distinct in both location and axonal trajectory to the CPA. The role of this novel descending sympathoexcitatory region in central cardiovascular regulation remains to be elucidated.

CHAPTER 2

CHAPTER 2

BLOOD PRESSURE REGULATION

I. Autonomic reflexes

The autonomic reflexes are the mechanisms that regulate BP in the short term. These reflexes act moment to moment, in seconds or minutes. They resulted from activation of peripheral receptors whose afferents projecting to the central nervous system via the glossopharyngeal and vagus nerves (Dampney, 1994). The processing of this afferent information, in the CNS, produces a consequent regulation of the autonomic efferent pathways leading to an adjustment of the cardiovascular parameters (heart rate, stroke volume, and vascular resistance) (Colombari *et al.*, 2001).

Central control of BP involves both the sympathetic and parasympathetic nervous system continuously controlling BP. The BP is controlled by the action of total peripheral resistance (TPR) and cardiac output (CO), and this depends on heart rate (HR) and stroke volume (SV) [BP = COxTPR, BP = HRxSVxTPR] (Loewy & Spyer, 1990a). The sympathetic nervous system increases stroke volume, heart rate and total peripheral resistance which promote an increase in BP. The parasympathetic nervous system acts mainly on heart rate decreasing it, but also acts on the contraction strength of the heart, decreasing stroke volume, which leads to a decrease in BP (Berne, 2004).

There are several reflexes involved in the modulation of sympathetic and parasympathetic activity, such as, the arterial baroreceptors, cardiopulmonary receptors and arterial chemoreceptors (Spyer, 1990; Sleight, 1991; Chalmers *et al.*, 1992; Dampney, 1994; Marshall, 1994; Vasquez, 1994; Machado *et al.*, 1997).

Reflexes are immediate and automatic responses of the body to an appropriate stimulus, without the intervention of consciousness or will. The basic unit of integrated reflex activity is the reflex arc (Ganong, 2005). This arc consists in 5 components: a sense organ, an afferent neuron, one or more synapses in a central integrating station or sympathetic ganglion, an efferent neuron, and an effector (Fig. 2-1; Ganong, 2005).

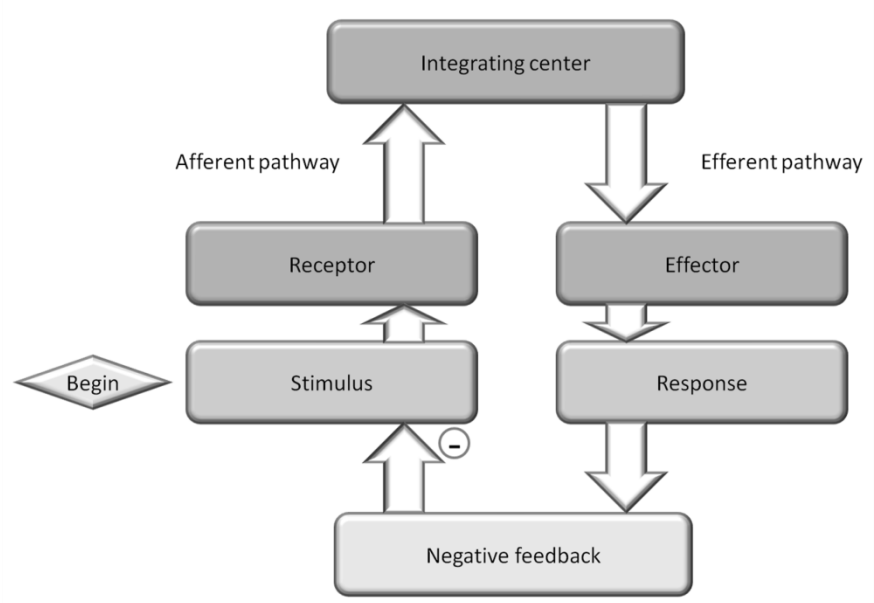


Figure 2-1. General components of a reflex arc that functions as a negative feedback control system. The response of the system has the effect of counteracting or eliminating the stimulus (negative feedback). Adapted from *Vander, Sherman, & Luciano's human physiology: the mechanism of body function*, 2004.

The simplest reflex arc is one with a single synapse between the afferent and the efferent neurons. Such arcs are monosynaptic, and reflexes occurring in them are monosynaptic reflexes. Reflex arcs in which one or more interneurons are interposed between the afferent and efferent neurons are polysynaptic, the number of synapses in the arcs varying from two to many hundreds (Ganong, 2005).

A stimulus is defined as a detectable change in the internal or external environment, such as a change in temperature, plasma potassium concentration, or blood pressure. A receptor detects the environmental change. A stimulus acts upon a receptor to produce a signal that is relayed to an integrating center. The pathway traveled by the signal between the receptor and the integrating center is known as the afferent pathway (Widmaier *et al.*, 2004).

An integrating center often receives signals from many receptors, some of which may respond to quite types of stimuli. Thus, the output of an integrating center reflects the net effect of the total afferent input; that is, it represents an integration of numerous bits of information. The output of an integrated center is sent to the last component of the system, a device whose change in activity constitutes the overall response of the system.

This component is known as an effector. The information going from an integrated center to an effector is like a command directing the effector to alter its activity. The pathway along which this information travels is known as the efferent pathway (Widmaier *et al.*, 2004). If the response produced by the effector causes a decrease in the magnitude of the stimulus that triggered the sequence of events, then the reflex leads to negative feedback and we have the typical homeostatic control system (Widmaier *et al.*, 2004).

The ***baroreceptor reflex*** is the main mechanism for adjusting blood pressure. The reflex is initiated by stimulation of baroreceptors that are nerve endings sensitive to stretching of the artery in each cardiac cycle. These receptors are found in reflexogenic areas along the high and low pressure vascular system and in the cardiac pump. In the vessels, the most relevant for blood pressure control are located in the carotid sinus at the bifurcation of common carotid artery, the aortic arch and mesenteric circulation (Fig. 2-2) (Agnoletti *et al.*, 1989). The communication between the aortic arch and the cardiovascular centers of the medulla is made via the vagus nerve and the carotid sinus communications is performed through the nerve of Hering, a branch of the glossopharyngeal nerve (Tresguerres, 2005). The arterial baroreceptors play a key role in the short-term adjustments of BP and are known to maintain BP in a normal range by actions on cardiac output and peripheral resistance as well as cardiac inotropism (Mancia *et al.*, 1979; deBoer *et al.*, 1987).

Also referred to as mechanoreceptors, the baroreceptors respond to distension and deformation that is imposed on the vessel, through local BP changes elicited by the phases of the cardiac cycle. This deformation causes, as an end result, a change in the frequency of nerve impulses that are carried to the nucleus tractus solitarius (NTS) (Donoghue *et al.*, 1984; Spyer *et al.*, 1984). The NTS located in the caudal dorsal medulla presents a functional and a viscerotopic organization and plays an important role in the modulation of autonomic efferent activity to the cardiovascular system (Paton, 1998; Silva-Carvalho *et al.*, 1998).

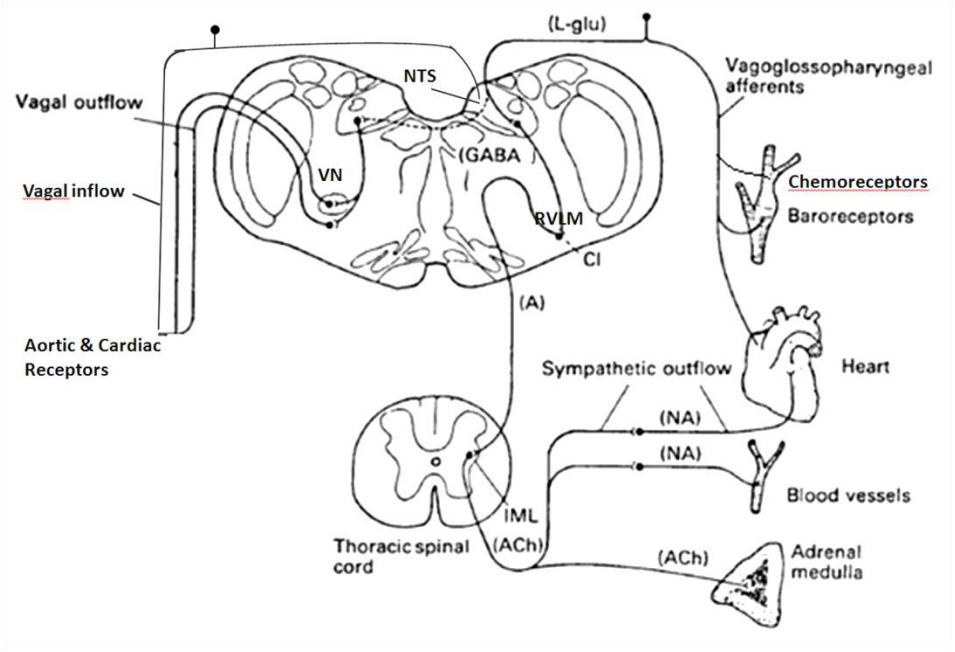


Figure 2-2. Location of the most prominent arterial baroreceptors. The arterial baroreceptors are strategically located for monitoring the blood pressure in the arteries that supply blood to brain (carotid sinus baroreceptor) and the rest of the body (the aortic arch baroreceptor, mesenteric baroreceptors and heart). Adapted from Ross *et al.*, 1985.

NTS is the primary central station for the reception of sensory information with origin in peripheral reflexogenic areas including the heart and vessels (Miura & Reis, 1969, 1972) and it is richly innervated by fibers arising from different brain nuclei belonging or not to the central autonomic network that are known to have an important role in cardiovascular control, including the parabrachial nucleus, the medial hypothalamus, and the amygdala (Crill & Reis, 1968; Miura & Reis, 1972; Loewy & McKellar, 1980; Colombari *et al.*, 2001). NTS neurones project into two groups of cells in the ventrolateral medulla located almost at the same antero-posterior level: 1) inhibitory neurons in the caudal ventrolateral medulla (CVLM), which project to premotor neurons in the rostral ventrolateral medulla (RVLM, sympathetic-excitatory neurons) which then project to the sympathetic preganglionic neurons of the intermediolateral (IML) cell column of the spinal cord, origin of pre-ganglionic sympathetic neurons) and; 2) neurons located in the nucleus ambiguous (NA) and dorsal motor nucleus of the vagus (DMNV), which contain the cell bodies of preganglionic neurons of the parasympathetic nervous system (PNS) (Krieger, 1964; Spyer, 1981) (Fig. 2-3).

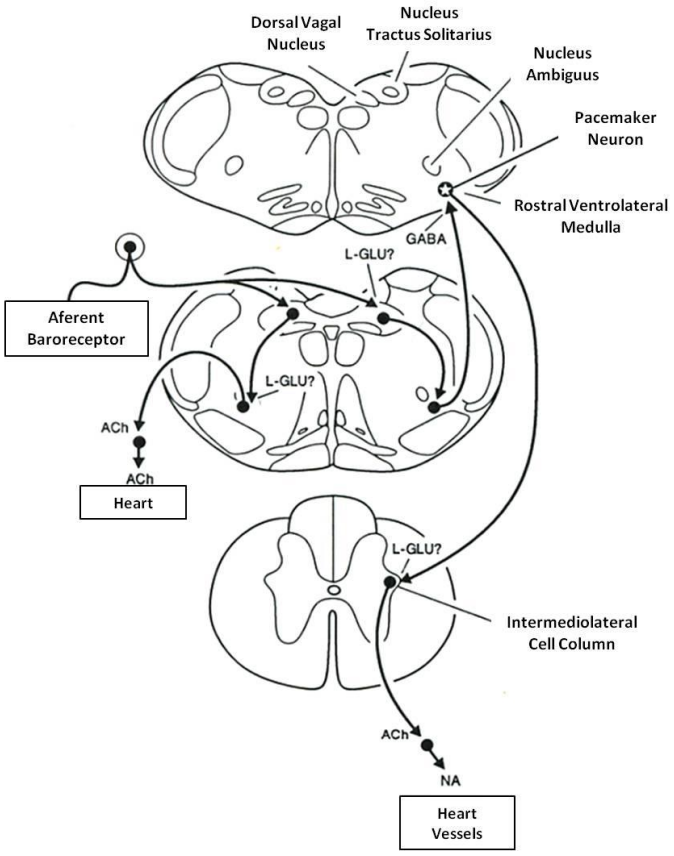


Figure 2-3 The general pattern of the baroreceptor reflex pathway, showing the relationship between the sensory receptors, the integrative brainstem regions and the motor innervations to the heart and blood vessels. AP; area postrema, CVLM; caudal ventrolateral medulla, DMNV; dorsal motor nucleus of the vagus, NA; nucleus ambiguous, NTS; nucleus tractus solitari, RVLM; rostroventrolateral medulla. Adapted from Loewy A and Spyer KM, 1990.

Changes in baroreceptor activity also affect breathing. As an example, *in vivo* studies on anesthetized vagotomized dogs, showed the carotid body chemoreceptor reflex response was eliminated by surgically excluding the carotid bodies from the carotid sinus baroreceptor area (Brunner *et al.*, 1982). These data showed that baroreceptor stimulation (by increasing carotid sinus pressure, whilst maintaining constant SBP) decreased respiration rate and increased end-tidal volume (Brunner *et al.*, 1982). Other studies have shown similar results (Grunstein *et al.*, 1975; Dove & Katona, 1985; Maass-Moreno & Katona, 1989). It has been suggested that changes in respiration in response to changes in carotid sinus pressure are due to increased/decreased firing of the type 1 large A-fibers (Hopp & Seagard, 1998).

Baroreceptors are also involved in vasopressin (VP) secretion (O'Donnell *et al.*, 1992), particularly in response to hypotension possibly due to the neuronal projections from the NTS to the PVN (Kawano & Masuko, 1996). When the baroreceptor reflex is activated by a reduction in BP, an increase in VP secretion is observed (Blessing & Willoughby, 1985). This has been shown also in human studies where plasma VP increased following reductions in MBP induced by ganglionic blockade (Baylis, 1987). In addition, in the dog, during hemorrhage, intact arterial baroreceptors were essential to maintain BP and VP secretion (Thrasher & Keil, 1998). Baroreceptors are vital for the reduction in the reflex increase in HR or renal SNA (RSNA) (in response to decreased MAP) following VP infusion (enhanced sympathoinhibitory effect) (Nishida & Bishop, 1992). However, in this situation, their action is reinforced by the facilitation of the chemoreceptor reflex at the NTS level due to the nature of the stimulus (Silva-Carvalho *et al.*, 1995a).

Several studies have discussed the role of the baroreceptor reflex in the regulation of long-term sympathetic activity, once a central resetting of the baroreceptor-sympathetic reflex may be an important component of the mechanism that causes persistent changes in renal sympathetic activity. However, little is known about the cellular mechanisms that can cause this resetting (Dampney *et al.*, 2005).

The ***chemoreceptors*** are highly specialized cells that can detect blood changes in the partial pressure of oxygen (pO_2), partial pressure of carbon dioxide (pCO_2) and blood pH. The peripheral chemoreceptors are more sensitive to changes in the pO_2 than changes in pCO_2 or pH whereas the central chemoreceptors respond primarily to changes in pCO_2 and pH (Berne, 2004).

The peripheral chemoreceptors are mainly located in the aortic and carotid bodies despite they can also be found in the mesenteric circulation (coeliac artery). The carotid bodies are placed bilaterally in the neck, at the bifurcation of the common carotid artery while the aortic bodies are disposed between the pulmonary artery and the aortic arch (Fig. 2-4) (Tresguerres, 2005). Carotid bodies are more sensitive to hypoxia and hypercapnia and they detect changes in blood gas tensions whereas aortic baroreceptors are more sensitive to anaemia, carboxi- hemoglobinaemia and systemic hypotension

being more involved in detecting changes in O_2 flow and in BP (Burgh Daly & Psychological, 1997). Thus, carotid bodies monitor ventilation/perfusion ratio and aortic bodies perform the reflex control of systemic vascular resistance.

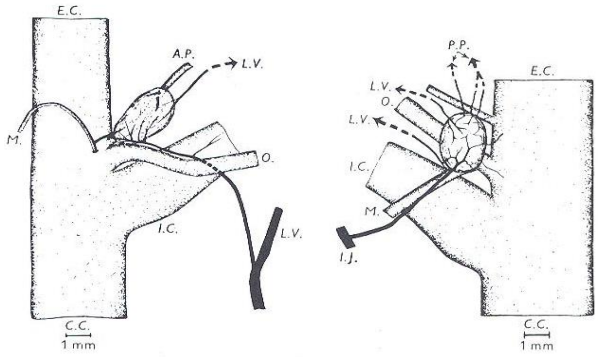


Figure 2-4. Ventromedial views of the left and right carotid bodies. EC: external carotid artery, IC: internal carotid artery; LV: left vagus. Adapted from Chungcharoen et al, 1953.

The central chemoreception initially was localized to areas on the ventral medullary surface at the area prostrema, however, there is substantial evidence that many sites participate in central chemoreception some located at a distance from the ventral medulla (Nattie & Li, 2012).

The chemoreceptor cells detect changes in the partial pressure of gases or pH, and through the vagus nerve or through the glossopharyngeal nerve (innervating, respectively, the aortic bodies and carotid bodies) send information to the nucleus tractus solitary (NTS) (Donoghue *et al.*, 1982).

The stimulation of the chemoreceptors causes at the central level, increase in the activity of NTS cells, some of them distinct but other similar from those activated by the baroreceptors. These cells simultaneously excite neurons in the NA and RVLM with consequent increase in sympathetic and parasympathetic tone (Fig. 2-5). The increase in the respiratory activity is due to various NTS neurons that when excited by chemoreceptor afferents have an inspiratory activity (Rocha, 1995).

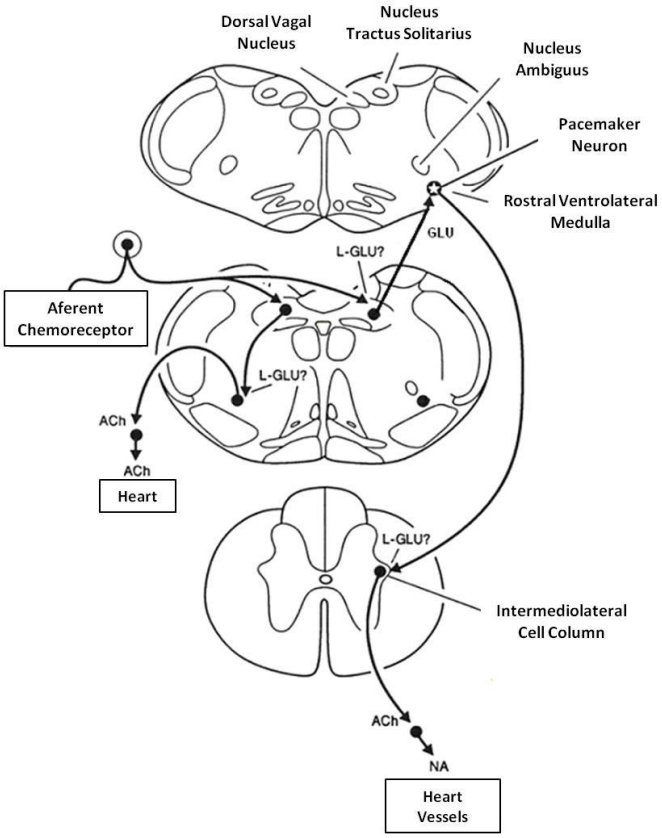


Figure 2-5. Schematic of the chemoreflex pathway, showing brainstem regions, SNS and PNS projections to the heart and blood vessels. A simplified overview of the chemoreceptor reflex showing the SNS and PNS arms. AP; area postrema, CVLM; caudal ventrolateral medulla, DMNV; dorsal motor nucleus of the vagus, NA; nucleus ambiguous, NTS; nucleus tractus solitari, RVLM; rostroventrolateral medulla. Adapted from Loewy A and Spyer KM, 1990.

Excitation of these receptors increases SNA mediating tachycardia, vasoconstriction and increased respiration rate (Loewy & Spyer, 1990a).

Activation of peripheral chemoreceptors results in ventilatory adjustments that are characterized by increased air flow volume, increased respiratory rate and increased breathing volume, thus playing an important role in the reflex control of ventilation (Heymans & Bouckaert, 1930). In addition to ventilatory responses, stimulation of the chemoreceptors also promotes changes in the cardiovascular system, in order to provide maintenance of the chemical composition of blood at optimal levels, as well as adequate blood perfusion to tissue.

Several studies suggest that peripheral chemoreceptors have a tonic excitatory influence on cardiovascular control, stimulate the sympathetic nervous system and thus contribute to the maintenance of BP levels and part of the total peripheral resistance.

In rats submitted to carotid denervation there was a significant reduction in renal sympathetic nerve activity. In rats submitted to acute hyperoxia (which induces the inactivation of chemoreceptors), there was a transient fall in BP and sympathetic activity. Selective removal of the activity of carotid chemoreceptors by compressing the artery that irrigates the carotid body also promotes a decrease in chronic levels of BP (Franchini & Krieger, 1992).

The tonic influence of chemoreceptors on the levels of BP and the primary reflex responses (bradycardia and hypertension) to stimulation of chemoreceptors with potassium cyanide were also demonstrated by Franchini and Krieger (Franchini & Krieger, 1992).

There are also volume (or stretch) receptors located in the right atrium and vena cava which respond to decreases in blood volume by reducing their firing rates and vice versa. The afferents of these receptors join the vagus nerve and terminate in the NTS, synapsing upon neurones which project from the NTS to the PVN (van Giersbergen *et al.*, 1992). This pathway is activated by changes in blood volume as small as 8-10% as shown electrophysiologically (Lovick & Coote, 1988) and by c-fos expression (a marker of neuronal activation) in the anaesthetised rat (Deng & Kaufman, 1995; Pyner *et al.*, 2002) and rabbit (Badoer *et al.*, 1997). Overall, the activation of these low pressure atrial receptors elicits an inhibition of sympathetic vasomotor tone and an increase of vasopressin secretion, which affects renal function.

Other reflexes potentially regulating blood pressure are the Bainbridge and the Bezold-Jarisch reflex. In Bainbridge reflex, blood pressure is indirectly regulated through heart rate changes. Accordingly, when right atrial volume increases, low-pressure stretch receptors (that is, receptors responding to stretch at the low pressures typical in the atria) initiate a reflex that increases heart rate through sympathetic nerves (Burgh Daly & Psychological, 1997). The Bainbridge reflex is not always active, depending its efficacy on the value of heart rate, being particularly efficient at lower rather the higher values of HR.

In this way, the Bainbridge reflex acts in opposition to the baroreceptor reflex which increases heart rate when the stretch is decreased in states of hypotension or hypovolemia (Bainbridge, 1915).The Bezold-Jarisch reflex is a chemically-sensitive cardiac reflex which strong depressor cardiovascular response of bradycardia and hypotension being evoked as a direct consequence of chemical stimulation of receptors in the ventricles or coronary circulation. The decrease in blood pressure is due both to the bradycardia and vasodilation caused by inhibition of sympathetic vasomotor activity and also modulates renin release and vasopressin secretion (Bezold *et al.*, 1867). Conversely, decreases in the activity of these inhibitory sensory receptors increase sympathetic activity, vascular resistance, plasma renin activity and vasopressin (Bezold *et al.*, 1867).

Ia. Autonomic reflexes and Hypertension

The autonomic nervous system (ANS) plays a crucial role in the control of BP and HR and is an important pathophysiological factor in the development of AHT.

In essential AHT, the cardiovascular homeostasis is partially lost or at least is maintained at a level of BP different from that of normotensive subjects (Sleight, 1991; Zanchetti & Mancia, 1991). The cause of this abnormality is not known, but the early alteration of baroreceptor control suggests that the ANS is deeply involved in the process (Bristow *et al.*, 1969; Eckberg, 1979). In fact, normotensive rats and spontaneously hypertensive rats (SHR) have similar baroreflex gain (and BP) at birth. In the weeks after birth, the baroreflex sensitivity increases rapidly in normotensive rats, but not in the SHR (Struyker-Boudier *et al.*, 1982).

One of the mechanisms associated with this autonomic imbalance is the reduced baroreflex sensitivity (BRS). The baroreflex is reduced or reset toward elevated blood pressure values in hypertension, blunting its ability to suppress the increased BP values promoted through the increased sympathetic activity (Mancia *et al.*, 1999; McCubbin *et al.*, 1956; Korner *et al.*, 1974; Eckberg, 1979; Grassi *et al.*, 1998). In the case of essential AHT the degree of decrease in baroreflex sensitivity for the control of heart rate is much higher than for the control of vascular resistance (Grassi *et al.*, 1998).

Above 160 mmHg increases in the intensity of responses are becoming smaller for the same BP increase, and this increase does not occur when the values exceed 200 mmHg. In addition, during the development of hypertension the baroreceptors adapt quickly to each tensional level, increasing the excitation threshold and reducing the maximum frequency of the discharge pulses.

Also most brainstem regions involved in controlling the baroreceptor reflex become more active (NTS and RVLM) or less active (CVLM) during the onset of hypertension, resulting in increased SNA (Smith & Barron, 1990; Grassi *et al.*, 1998; Colombari *et al.*, 2001).

In addition, the severity of the baroreflex sensitivity change is correlated with the severity of AHT. However, is not yet well understood whether this decrease - also observed in pre-hypertensive patients - precedes or participates in the development of AHT (Eckberg, 1979).

Another study showed that slow breathing reduces blood pressure and enhances baroreflex sensitivity in hypertensive patients, suggesting a potentially beneficial effect in hypertension (Joseph *et al.*, 2005).

The baroreflex activity can be modulated by several peptides of the RAAS (Averill & Diz, 2000). Studies in rats, rabbits and dogs showed the inhibitory action of Ang II on the baroreflex in AHT after peripheral or central administration (in the medulla oblongata). Ang II, modulates the baroreceptor reflex by diminishing the sensitivity of the reflex and shifting the operating point for regulation of sympathetic outflow to higher blood pressures (Dampney, 1994; Phillips & Sumners, 1998; Averill & Diz, 2000).

The central administration of angiotensin I converting enzyme (ACE) inhibitors, non-selective peptidergic antagonists and antagonists of AT1 receptors in SHR rats produces a decrease in BP (Phillips & Sumners, 1998; Averill & Diz, 2000). So, the decrease in the central hyperactivity of the RAAS leads to a decrease in BP. The mechanisms by which the hyperactivity of the RAAS causes AHT are the same as those involved in the antihypertensive effect produced by central administration of Ang II, i.e., stimulation of the release of vasopressin, SNS activation and the inhibition of the baroreflex. This suggests that the Ang II is involved not only in shifting the operating band of the baroreflex (change of set point), but also in the decrease in the baroreflex gain.

Other studies have shown that the central infusion of a selective antagonist of Ang-(1-7), A-779, produces a significant decrease in baroreflex, whereas the infusion of a selective antagonist of the AT1 receptor improves the baroreflex sensitivity (Oliveira *et al.*, 1996; Heringer-Walther *et al.*, 2001). It was also been shown an increase in baroreflex sensitivity in SHR rats after intracerebroventricular administration of captopril (ACE inhibitor) (Phillips & Sumners, 1998; Averill & Diz, 2000).

Furthermore, it is known that the effect of Ang II on the baroreflex is independent of the rise on BP caused by this peptide. Several studies have shown that even when it prevents the decrease in BP after central administration of angiotensin-converting enzyme (ACE) inhibitors, there are changes in baroreflex sensitivity towards normal values (Averill & Diz, 2000). This observation leads to a very important suggestion: the effect of RAAS or Ang II on the baroreflex should be made in the central nervous system.

Other mechanism that may be involved in the autonomic imbalance is the chemoreflex activation in essential hypertension, which can be an additional mechanism responsible for the increase in sympathetic activity (Somers *et al.*, 1988).

It is known that the SHR rats have an increased chemoreflex activity (Przybylski, 1981; Hayward *et al.*, 1999). It has been observed that young hypertensive animals had increased pH, and pO2 and pCO2 reduction in blood when compared to normotensive animals (Hayward *et al.*, 1999). Additionally, SHR rats have an increased respiratory volume than the normotensive rats (Fukuda *et al.*, 1987; Hayward *et al.*, 1999). This suggests that the changes found in the chemical composition of the blood of hypertensive animals would result from hyperventilation, possibly induced by the hyperactivity of the peripheral chemoreceptors in these animals.

In fact there are several studies that correlate the AHT with the activity of the peripheral chemoreceptors (Przybylski, 1981; Habeck, 1991; Abdala *et al.*, 2012). It has been shown through recordings of carotid sinus nerve activity, that SHR have an increased chemoreceptors sensitivity to hypoxia compared to normotensive rats (Fukuda *et al.*, 1987). The same was observed in humans with essential hypertension or pre-hypertensive patients, once there was a significantly increase in ventilatory, airway

occlusion pressure and blood pressure response to hypoxia in the hypertensive subjects (Trzebski *et al.*, 1982; Somers *et al.*, 1988).

Other studies have shown that hyperoxia induced deactivation of carotid body chemoreceptors, reduces sympathetic activity in hypertensive patients, but it does not affect BP (Seals *et al.*, 1991; Seals & Reiling, 1991). The maintenance of BP values can be explained by the direct, vasoconstrictive effect of hyperoxia, which offsets diminished sympathetic activity (Sinski *et al.*, 2014). In a recent study it was compared the effect of acute hyperoxia on hemodynamic parameters between hypertensive and normotensive subjects and they confirm that deactivation of carotid body chemoreceptors can acutely decrease blood pressure in humans (Sinski *et al.*, 2014).

Human and animal studies have also shown that in the AHT, the peripheral chemoreceptors (especially in the carotid bodies) exhibit morphological, biochemical and functional changes, which might be correlated with the genesis of hypertension (Edwards *et al.*, 1971; Heath *et al.*, 1986; Habeck, 1991; Abdala *et al.*, 2012).

These changes in chemoreflex sensitivity in SHR may be due to narrowing of the lumen of the artery to the carotid body, possibly resulting from atherosclerosis in the in the carotid sinus region (Habeck, 1991), changes in the capillaries of the carotid corpuscles of SHR (Smith *et al.*, 1984) or due to an increase in size of the carotid body (Habeck *et al.*, 1981; Alho *et al.*, 1984).

II. Humoral factors

The hormonal regulation of BP is exerted by circulating vasodilators, such as arterial natriuretic peptide (ANP), prostaglandins and kinins and vasoconstrictors, such as vasopressin, catecholamines and angiotensin.

Bold and collaborators showed that the heart has an endocrine role in the production of ANP (de Bold *et al.*, 1981). In fact, the heart release this peptide not only by atrial distension, but also by ventricular distension and neurohumoral stimuli (Edwards *et al.*, 1988). Various ANP actions contribute to the regulation of blood pressure, including its vasodilator, diuretic and potent natriuretic effect, as well as their action in the modulation of the activity of the RAAS (Maack *et al.*, 1985; Chen, 2005). Thus, under normal conditions, the release of the ANP leads to an increased renal excretion of sodium and potassium in response to increases in BP. Moreover, in healthy subjects, the administration of low doses of ANP strikingly increases the urinary excretion not only of sodium but also of the primary solutes retained in chronic renal failure, such as urea, potassium, and phosphate, with no systemic effect (Richards *et al.*, 1985; De Nicola *et al.*, 1993; Conte *et al.*, 1997; De Nicola *et al.*, 1997). The diuretic effectiveness of low-dose ANP in normal subjects appears to be dependent on the attainment of plasma ANP levels immediately above the physiological range (Richards *et al.*, 1985; De Nicola *et al.*, 1993; Conte *et al.*, 1997; De Nicola *et al.*, 1997).

One of the important clinical features of increased SNS activity is sodium and water retention. Clonidine tends to stimulate diuresis and natriuresis by mechanisms that involve actions on the renal tubule to modulate the actions of vasopressin (Pettinger *et al.*, 1987) or through the release of the ANP, which stimulates cGMP production in different target cells leading to vasodilation and natriuresis (Mukaddam-Daher & Gutkowska, 2000). Previous work (Baranowska, 1987; Chen *et al.*, 1989; Gutkowska *et al.*, 1997; Mukaddam-Daher *et al.*, 1997) demonstrated that in addition to sympathoinhibition, α2-adrenergic receptors play a role in the cardiac release of ANP. *In vivo* administration of clonidine or its peripherally acting analogue induces dose-related increases in plasma ANP levels and results in diuresis and natriuresis (Baranowska, 1987;

Chen *et al.*, 1989; Gutkowska *et al.*, 1997; Mukaddam-Daher *et al.*, 1997). Also ANP is inhibited by α2-adrenergic receptor antagonists (Baranowska *et al.*, 1987).

Prostaglandins and kinins constitute a major blood pressure-regulating system which opposes the effects of circulating vasoconstrictor hormones, such as angiotensin, vasopressin and catecholamines, and moderate the release of norepinephrine from vasoconstrictor nerves (Kahn *et al.*, 1973; McGiff & Quilley, 1980). Important interactions of prostaglandins and kinins that can decrease BP occur within the kidney and blood vessels where they contribute to the regulation of extracellular fluid volume and vascular reactivity (McGiff & Quilley, 1980).

Vasopressin (arginine vasopressin, AVP or antidiuretic hormone, ADH) is a peptide hormone formed in the hypothalamus, then transported via axons to, and released from the posterior pituitary. Vasopressin has a potent vasoconstrictor effect and, in cases of severe bleeding, vasopressin plays a fundamental role since it markedly increases the reabsorption of water from renal tubules, thereby increasing the volume (Guyton & Hall, 2006).

Other vasoconstrictors are the catecholamines, epinephrine and norepinephrine, that are originated from two sources. Epinephrine is released upon activation of preganglionic sympathetic nerves that innervate the adrenal medulla and occurs during times of stress (e.g., exercise, heart failure, haemorrhage, emotional stress or excitement, pain). The primary source of circulating norepinephrine is spillover from sympathetic nerves innervating blood vessels. Therefore, at times of high sympathetic nerve activation, the amount of norepinephrine entering the blood increases dramatically. Both circulating catecholamines are released by the adrenal medulla (Klabunde, 2012).

The RAAS plays an important role in regulating blood volume and systemic vascular resistance, which together influence cardiac output and BP. Briefly, in the RAAS, the renin is released by the kidney into the circulation where it cleaves angiotensinogen to angiotensin I, which is cleaved by angiotensin converting enzyme (ACE) to angiotensin II (Ang II) (Veerasingham & Raizada, 2003). Ang II causes vasoconstriction increasing total peripheral resistance, increases water retention due to aldosterone release which

increases kidney sodium reabsorption collectively acting to increase BP (Bader & Ganten, 2002, 2008).

The hormonal system is affected by the action of endothelin (ET) peptides and their receptors. So ET affects natriuretic peptides, aldosterone, catecholamines, and angiotensin, being intimately involved in the physiological control of systemic BP and body sodium (Na) homeostasis. ET also directly regulates cardiac output, central and peripheral nervous system activity, renal Na and water excretion, systemic vascular resistance and venous capacitance (Kohan *et al.*, 2011).

CHAPTER 3

CHAPTER 3

RATIONALE, HYPOTHESIS, METHODS AND RESULTS

I. Overall purpose of the present PhD thesis

Hypertension (AHT) is a recognized and important risk factor for cardiovascular disease. Its prevalence has been increasing and it is estimated that about one billion people suffer from AHT worldwide. However, for the great majority of patients, the treatment decisions are just clinical and the therapeutic strategy it is only symptomatic as the aethiology of the disease is unknown. Among the different origins and mechanisms of deregulation that have been discussed, the neurogenic nature of this cardiovascular dysfunction has been postulated by several authors.

The central nervous system (CNS) plays an important role in regulating blood pressure instant-by-instant, but its contribution to chronic blood pressure regulation is still not clear, in particular in allostatic conditions. Several studies suggest that sympathetic nervous system (SNS) is a major factor on the onset, development and maintenance of several cardiovascular pathologies including essential hypertension. The continuous sympathoexcitation observed in the hypertensive state can result firstly from a protective defense reaction through negative feedback mechanisms which would be transformed in a deleterious overprotection relying on long term neurohumoral responses with major maladaptive consequences. This inappropriate change and/or maintenance of activity can be originated at any level of the autonomic reflex arc including the brain centres which integrate sympathetic activity.

Therefore, *the overall purpose* of this PhD thesis is to target central sympathetic nuclei to address the mechanisms putatively involved in the generation of sympathetic activation in hypertensive conditions of unknown origin.

With the fulfilment of this goal is expected to:

i) Identify regions in the central nervous system from where a persistent simultaneous decrease in arterial blood pressure and sympathetic activity due to genetic modification of their cells excitability can be evoked;

ii) Evaluate hypertensive target organs characteristics upon the central evoked decrease of arterial blood pressure and sympathetic output to conclude on their reverse remodelling process;

iii) Define the relationship between cardiorespiratory reflexes regulation - baroreceptor and chemoreceptor reflex - hypertensive target organs condition and cardiovascular risk;

iv) Outline new physiological roles in neurogenic hypertension for the target brain areas;

v) Discuss autonomic modulation with particular focus on the integrative centres of the autonomic reflex arc, as a new therapeutic strategy for neurogenic hypertension.

II. Specific aims of the project

The work flow of the project was divided in 2 parts, according to the specific aim.

In the 1st part, the aim was to decrease the sympathetic tone by affecting brain sympatho-excitatory regions. For that, the activity of PVN, RVLM and MPCA neurones was depressed by the over-expression of a potassium channel in order to reduce neuronal membrane excitability.

In the 2nd part, through the elicited decrease of sympathetic activity and BP evoked by potassium channels overexpression, the effect of a putative autonomic therapeutic was evaluated on target organs. For that, through RT-PCR, gene expression changes in hypertensive target organs were analyzed.

III. Exploring the hypotheses under study

Hypothesis 1 - Will a chronic reduction of neuronal excitability within the paraventricular nucleus of the hypothalamus evoke a persistent reduction of arterial blood pressure and sympathetic activity with impact in respiratory, baro and chemoreceptor function?

Hypothesis 2 - What is the role of rostral ventrolateral medullary and medullar pressor cervical neuronal activity in the long term maintenance of high blood pressure values, sympathoexcitation and baroreflex blunting in the hypertensive condition?

Hypothesis 3 – Will the chronic depression of brain sympatho-excitatory regions activity induce major signalling changes in hypertensive target organs condition?

HYPOTHESIS 1

The paraventricular nucleus of the hypothalamus is a rather complex area composed by several groups of neurons including autonomic neurons which play an important role on cardiovascular control, both on physiological and pathological conditions. Through reciprocal connections with other areas of the central autonomic network, PVN is involved in the integration of autonomic and endocrine responses that regulate visceral functions. The PVN receives sensory information of neuronal and humoral origin which is sent, after integration, to lower autonomic centres located at the brainstem and spinal cord. In this way, PVN is able to exert multiple autonomic effects including influencing sympathetic activity. In accordance, the following working hypothesis was designed:

Will a chronic reduction of neuronal excitability within the paraventricular nucleus of the hypothalamus evoke a persistent reduction of arterial blood pressure and sympathetic activity with impact in respiratory, baro and chemoreceptor function?

1. INTRODUCTION

Essential arterial hypertension (EHT) has now reached pandemic proportions with an estimated one billion sufferers worldwide. The pathogenesis of EHT is multi-factorial and not completely understood but there is clear evidence that chronic elevation of sympathetic nervous system (SNS) activity is a major contributor to the onset, development and maintenance of the hypertensive state (Grassi, 2004b; Guyenet, 2006; Fisher & Paton, 2012). In fact, the increase of sympathetic outflow to the heart results in increased cardiac output and neurally mediated vasoconstriction leading to elevated blood pressure values (Schlaich *et al.*, 2012). In white coat and borderline hypertensive patients, sympathetic nerve activity to the arterioles supplying skeletal muscle is already raised compared to healthy individuals (Grassi, 2004a; Smith *et al.*, 2004). Excessive sympathetic activity may contribute to vascular smooth and cardiac muscle hypertrophy, brain hypoperfusion and inflammation, and becomes a major target to control in neurogenic hypertension (Zubcevic *et al.*, 2011).

The evaluation of sympathetic activity can be achieved indirectly by applying mathematical tools such Fast Fourier Transform (FFT) to blood pressure signals (M Malik, 1996). A power spectrum is, then, generated where the low frequencies (LF) represent predominantly sympathetic activity and high frequencies (HF) are related with parasympathetic tonus and respiration (Radaelli *et al.*, 1994; M Malik, 1996; Furlan *et al.*, 2000). These mathematical results have been addressed in several studies which have suggested that sympathetic activity is a critical determinant of blood pressure fluctuations at a frequency range which is slower than the rate of respiration (Japundzic *et al.*, 1990; Cerutti *et al.*, 1991; Malliani *et al.*, 1991).

Located in the hypothalamus, the paraventricular nucleus (PVN) is a major sympatoexcitatory area, that becomes more active under conditions of hypertension such as in the spontaneously hypertensive rat (SHR) model (Allen, 2002). Some authors have referred to this region as a command nucleus providing feed forward excitatory synaptic drives to coordinate lower brainstem cardiovascular and respiratory motor activity (Dampney *et al.*, 2005). PVN activation promotes an increase in sympathetic output and a pressor effect mediated via direct and indirect projections (via rostral ventrolateral medulla, RVLM), to the spinal cord (Caverson *et al.*, 1984; Shafton *et al.*, 1998; Pyner & Coote, 2000; Hardy, 2001).

Both electrical stimulation and chemical manipulation of PVN neurons with bicuculline (a $GABA_A$ receptor antagonist) or glutamate elevated sympathetic nerve activity causing hypertension in anesthetized and conscious rats (Kannan *et al.*, 1989; Zhang *et al.*, 2002). In contrast, acute inhibition of the PVN with GABA or muscimol reduces the blood pressure and sympathetic nerve activity in SHRs (Allen, 2002). PVN lesions or the transection of the brain caudal to the hypothalamus promotes a decrease in blood pressure in SHRs but not in Wistar Kyoto (WKY) rats (Yamori & Okamoto, 1969; Goto *et al.*, 1981; Ciriello *et al.*, 1984; Herzig *et al.*, 1991; Takeda *et al.*, 1991).

Long-term manipulation of neurone excitability can be performed by expressing a human inwardly rectifying potassium channel (hKir2.1) under the control of a selective neuronal promoter such as synapsin (Duale *et al.*, 2005b; Duale *et al.*, 2007). Inwardly rectifying potassium channels, like Kir2.1, are endogenously expressed in rat brain and have recently been over expressed as a means to reduce neuronal membrane excitability (Yu *et*

al., 2004; Duale *et al.*, 2007; Mizuno *et al.*, 2007; Okada & Matsuda, 2008; Yoon *et al.*, 2008; Howorth *et al.*, 2009). Their long-term expression can be achieved by the use of lentiviral vectors (LVV) derived from human immunodeficiency virus (Coleman *et al.*, 2003). Therefore, using a LVV to over-express hKir2.1 channels within the PVN we asked what long term influence does this nucleus have on the control of blood pressure, heart rate, sympathetic activity and respiration in the SHRs as well as homeostatic reflex control mechanisms?

2. METHODS

All the experimental procedures were in accordance with the European and Portuguese Law on animal welfare and had the approval of the ethics committee of the Faculty of Medicine, University of Lisbon, Portugal. Male Wistar–Kyoto rats (n =15) and SHRs (n =15) were used, aged 12 weeks and weighing 363±8 g. Animals, synchronized to a 12 h–12 h light–dark cycle (light on at 07.00 h and light off at 19.00 h), were housed individually and allowed to freely move in standard plastic cages. Food and water were available ad libitum.

2.1 Viral vector construction and validation

Lentiviral vector construction was based on previous studies (Waki et al. 2003; Duale et al. 2007). Briefly, LVV-eGFP, used for the sham-treated group, was a mix of LVTREtight-GFP 5.7×109 IU and LV-Syn-Eff-G4BS-Syn-Tetoff 6.2×109 IU in a ratio 1:4. This binary system expresses enhanced green fluorescent protein (eGFP). The LVV-hKir2.1 is a mix of LV-TREtight-Kir-cIRES-GFP 5.4×109 IU and LV-Syn-Eff-G4BS-Syn-Tetoff 6.2×109 IU in a ratio 1:4, which expresses eGFP and expresses human inwardly rectifying potassium channels (hKir2.1) in neurones. Validation of transduction efficacy and transgene expression was assessed as described previously by Duale et al. and included mRNA expression, immunocytochemical and electrophysiological data (Duale *et al.*, 2007).

2.2. Microinjection sites

Initially, we fine tuned our stereotaxic coordinates for bilateral PVN microinjections in 5 SHR and 5 WKY rats anaesthetised with sodium pentobarbitone (60mg/Kg, IP, Hikma Pharmaceuticals, London, UK). Bilateral microinjections (0.05µl) of LVV-eGFP were performed. Using fluorescence microscopy and histological reconstruction we determined the correct coordinates for PVN injections and the amount of LVV-eGFP, needed to limit transduction to the confines of the PVN.

2.3. Surgery

SHRs were divided into 2 groups according to the microinjection content: LVV-hKir2.1 (n=8) and LVV-eGFP (n=7). A control group of WKY rats, matching age, sex and number of individuals, underwent the same surgical and experimental protocol.

a) Implantation of telemetry probes

Rats were implanted with radio-telemetry probes (DSI, St. Paul, Minnesota, MN, USA) in the abdominal aorta under general anaesthesia (sodium pentobarbitone, 60mg kg^{-1}, I.P., Hikma Pharmaceuticals).Animalswere allowed to recover for 15 days. Similar anaesthetic and surgical protocols were applied to WKY rats (n =15).

b) Bilateral microinjection in the PVN

Two weeks after the probes were implanted, SHRs (n =8) and WKY rats (n =8) under general anaesthesia (sodium pentobarbitone, 60mg kg−1, I.P.,Hikma Pharmaceuticals) were placed in a stereotactic frame (Kopf Instruments, Tujunga, CA, USA), and a craniotomy was performed using our previously determined co-ordinates for LVVhKir2.1 microinjections (0.05 µl) into the PVN (Bregma, −1.6 mm; Lateral, ±1.41 mm; Deep, 7.4 mm; pipette angle, 10 deg to bregma; (Paxinos & Watson, 1986). Sham treated rats were microinjected in the same region with LVV-eGFP (SHRs, n =7; and WKY rats, n =7). All microinjections were performed bilaterally. Animals of all groups were allowed to recover

and monitored by telemetry for 60 days. Heart rate (HR) and blood pressure [BP; systolic (SBP), diastolic and mean] were recorded continuously.

2.4. Metabolic Evaluation

Rats were housed for 24h in metabolic cages to evaluate food and fluid intake, urine and feces production and body weight. Measurements were performed before and 59 days after each microinjection.

2.5. Cardio-respiratory reflex evaluation

At 60 days, animals were anesthetised (sodium pentobarbitone, 60mg/Kg, IP, Hikma Pharmaceuticals). The trachea was cannulated below the larynx to record tracheal pressure (TP). The femoral and carotid arteries and femoral vein were cannulated. Rectal temperature was maintained at 38±1°C by a servo-controlled heating blanket. The electrocardiogram (ECG) was recorded with the use of needle electrodes inserted into the limbs and HR was derived from the ECG. Baroreceptor and peripheral chemoreceptor reflexes were activated twice with an interval of 5 minutes between each stimulation. Baroreceptor reflex was stimulated by phenylephrine (0.2ml, 25µg/ml i.v.; Sigma Aldrich). Peripheral chemoreceptor reflex was stimulated with lobeline (0.2ml, 25µg/ml, Sigma Aldrich) injected retrogradely into the bifurcation of the common carotid artery. HR, BP (systolic, diastolic and mean) and respiratory rate (RespR) were recorded continuously thought the experiment.

2.6. Histology and immunochemistry

Animals were terminally anesthetized and immediately perfused transcardially with phosphate-buffered saline (PBS; 0.1M; pH 7.4) followed by 4% paraformaldehyde (0.1M; pH 7.4). The brain was removed and placed for 48 h in 15% (w/v) sucrose solution. Coronal sections (18 µm) were cut on a microtome and mounted on slides. The pipette tip location and the microinjection diffusion in the PVN were examined and documented. The microinjected contents (LVV-hKir2.1 or LVV-eGFP) containing e-GFP allowed an

estimation of virus dispersion. eGFP-labeled fluorescent regions were identified using an epifluorescence microscope and plotted on standardized sections from the Paxinos and Watson atlas (Paxinos & Watson, 1986).

2.7. Western blot analysis

The expression of hKir2.1 in the PVN was analysed by Western blot 60 days after the microinjection of LVV-hKir2.1 (n =8) or LVV-eGFP in SHRs (n =7). The PVN was dissected fromboth groups and homogenized by sonication in ice-cold RIPA buffer (Sigma, St. Louis, MO, USA) supplemented with a cocktail of protease inhibitors (complete mini; Roche). Proteins were extracted from the homogenates by centrifugation at 5000g for 10 min at 4ºC, and protein concentration was determined with a Bio-Rad DC Protein Assay kit. Proteins were resolved by electrophoresis on a 10% Tris–glycine SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA,USA).Membranes were blocked with 5% milk in Tween/Tris-buffered saline and incubated overnight at 4ºC with rabbit anti-hKir2.1 polyclonal antibody (Abcam, Cambridge, UK). After washing, membranes were incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit antibody (Bio-Rad, Hercules, CA, USA), and immunoreactive proteins were detected by Immobilon Western Chemiluminescent HRP Substrate (Millipore, Bedford, MA, USA) and visualized using Curix 60 (AGFA, Greenville, SC, USA).Membranes were stripped with 0.1 M glycine (pH 2.2) and reprobed with the α-tubulin antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for loading control.

2.8. Data acquisition and analysis

Telemetric data were acquired at 1KHz and analyzed with suitable software (LabChart6, Powerlab, ADInstruments). Mean values of HR, BP (systolic, diastolic and mean) and RespR were extracted.

a) Baroreceptor and chemoreceptor reflex

The baroreceptor reflex gain (BRG) was quantified calculating ΔHR/ΔBP (bpm.mmHg^{-1}). Chemoreceptor (ChR) reflex was calculated through the RespR derived from the tracheal pressure before and after stimulation with lobeline: $\Delta ChR = RespR_{lobeline} - RespR_{basal}$. BP and HR were also evaluated.

b) Analysis of BP and HR variability

Systolic BP and RR interval data were analyzed (period of 3 minutes) in the frequency domain (Fast Fourier Transform, FFT), using the in-house software Fisiosinal (Tavares, 2011b), to evaluate sympathetic (Low Frequency band, LF, 0.15-0.6Hz of SBP) and parasympathetic (High Frequency band, HF, 0.6-2.0Hz of HR) activity over time.

c) Circadian light/dark heart rate and blood pressure profile

Mean BP and HR values were calculated using the continuous telemetric data and compared between light (07.00–19.00 h) and dark phases (19.00–07.00 h).

2.9. Statistical analysis

Comparisons between groups for the same period and also comparisons within the same group, before and after the microinjections were performed. For the statistical analysis, Student's t test for paired data and ANOVA for comparisons between groups were used. All data were expressed as mean ± SEM and passed the normality test. Significance was taken as $P < 0.05$.

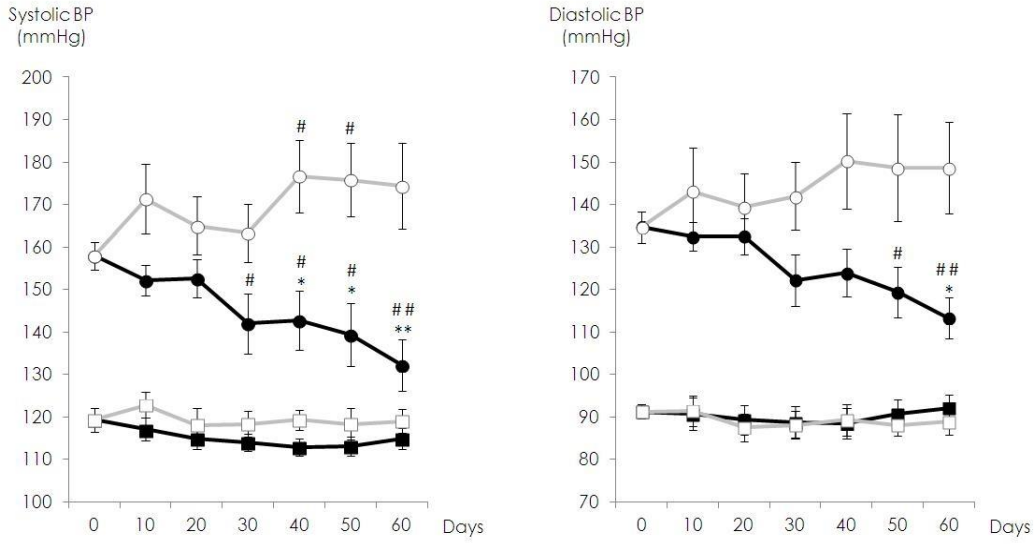
3. RESULTS

3.1 Effect of LVV-hKir2.1 or LVV-eGFP microinjection on 24h mean values of blood pressure, heart rate and respiration

Basal BP values (recorded before microinjections) in conscious SHRs (n=15) were 158±3 mmHg for systolic BP, 135±4 mmHg for diastolic BP and 142±3 mmHg for mean BP, being

significantly higher than those for WKY rats (119±3, 91±2, 101±1 mmHg respectively; n=15; p<0.0001). SHRs showed a higher baseline respiratory rate than WKY (77±5 vs 61±4 cpm, p<0.05) as well as a lower HR (311±5 and 367±9 bpm, for SHR and WKY rats, respectively, p<0.0001).

Thirty days after LVV-hKir2.1 microinjection, a significant BP decrease (p<0.05) was first observed but in order to evaluate its persistence, animals were monitored for a further 30 days. At the 60th day after lentiviral microinjection, SHRs values for systolic, diastolic and mean BP were 132±6, 113±5 and 120±5mmHg, corresponding to a decrease in pressure of 26mmHg, 22mmHg and 22mmHg, respectively (p<0.01, Figure 3-1). These BP changes were accompanied by a lowering of HR (295±3 bpm, p=0.099) but RespR remained unchanged. The decreased BP and HR values approached those of normotensive animals. At the same time, SHR LVV-eGFP were showing increased values of systolic (174±10 mmHg, p>0.05), diastolic (149±11 mmHg, p>0.05) and mean BP (157±10 mmHg, p>0.05) together with a significantly HR decreased (285±6 bpm, p<0.01). This profile of BP and HR changes was expected and is a consequence of maturation. In contrast, no significant changes in BP, HR and RespR were observed in WKY rats during the 60 days duration of the experimental protocol.



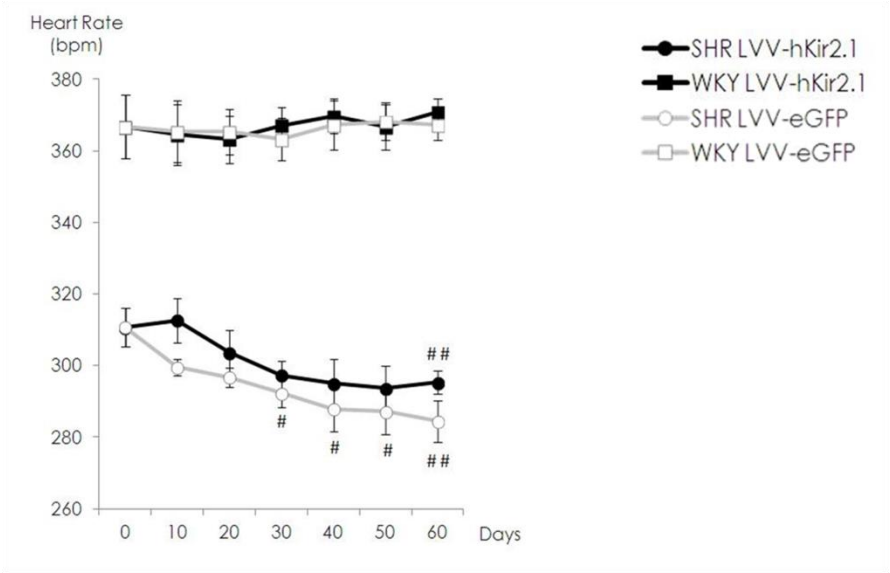


Fig. 3-1 – Effect on systolic, diastolic blood pressure and heart rate before (0 days) and after microinjection of LVV-hKir2.1 (n=7) or LVV-eGFP (n=7). The asterisks denote statistically significant differences between SHR LVV-hKir2.1 and SHR LVV-eGFP groups and the cardinals denote statistically significant differences within the group; *,#p < 0.05; **,##p < 0.01.

3.2. Effect of LVV-hKir2.1 microinjection on sympathetic output measured indirectly

SHRs showed putative evidence for an overall decrease of cardiovascular autonomic outflow at 60 days after LVV-hKir2.1 microinjection when compared with basal autonomic output at day 0. In fact, by using FFT applied to systolic BP and RR intervals, a decrease of LF_{sBP}/HF_{RR} ratio (from 0.07±0.02 to 0.04±0.01 $mmHg^2.ms^{-2}$; p>0.05) was observed, mainly due to a strong decrease in sympathetic output expressed by LF_{sBP} band power (from 0.79±0.13 to 0.42±0.09 $mmHg^2$, p<0.05). In SHR the basal HF_{sBP} (0.75±0.10 $mmHg^2$) was first reduced at 40 days and persisted until 60 days (0.33±0.10 $mmHg^2$; p<0.05) after LVV-hKir2.1 but unchanged in the LVV-eGFP group (0.82±0.38 $mmHg^2$). Interestingly, LF SBP was significantly reduced by 20 days after LVV-hKir2.1 microinjection and occurred before the fall in SBP. In contrast, at 60 days the LF_{SBP}/HF_{RR} ratio for SHR LVV-eGFP was 0.08±0.03 $mmHg^2$ ms^{-2} and the LF was 0.86±0.21 mmHg2 (P >0.05). The variations of mean LF_{SBP} and LF_{SBP}/HF_{RR}, at 10 day intervals for each SHR group, are depicted in Fig. 3-2. For WKY rats in basal conditions, the LF_{SBP} and LF_{SBP}/HF_{RR} ratio were 3.23±0.36 $mmHg^2$ and 0.43±0.14 $mmHg^2$ ms^{-2}, respectively. No significant changes in LF and LFSBP/HFRR ratio were observed for WKY LVV-hKir2.1 (3.11±0.44 $mmHg^2$ and 0.40±0.23 $mmHg^2$ ms^{-2},

respectively) and WKY LVVeGFP rats (2.56±0.48 $mmHg^2$ and 0.22±0.08 $mmHg^2$ ms^{-2}, respectively).

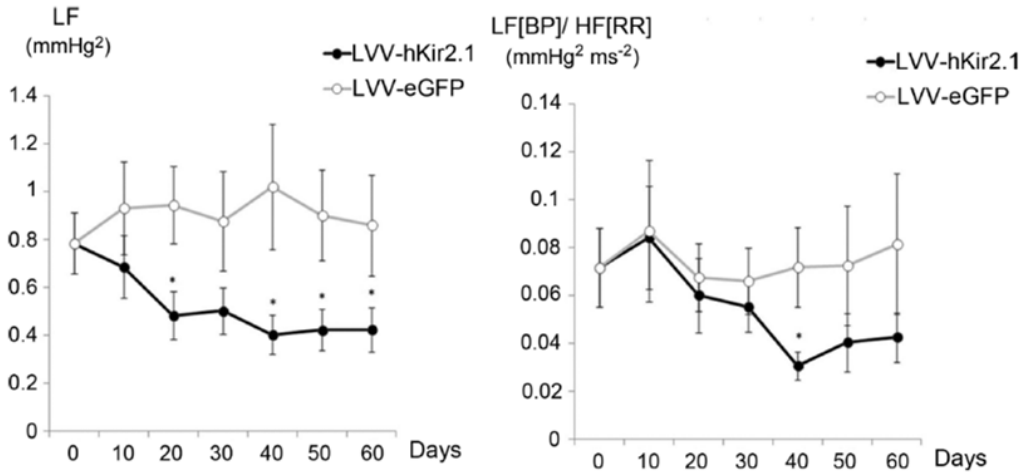


Fig. 3-2 – Mean (±SEM) LF and LF(BP)/HF(RR) before (0 days) and 10 days intervals after the microinjection of LVV-hKir2.1 or LVV-eGFP in SHR. Note that the fall in LF SBP occurred a week before the fall in SBP suggesting a causative association. The asterisks denote statistically significant differences between groups; *p < 0.05.

3.3. Arterial baroreflex gain (BRG) and peripheral chemoreflex responsiveness

The injection of phenylephrine triggered, in all animal groups, a progressive increase in mean BP, which was accompanied by a progressive reduction in HR. In SHR, BRG increased significantly after LVV-hKir2.1 microinjection approaching the values of the normal controls. SHR LVV-hKir2.1 group had a higher BRG in comparison to the SHR LVV-eGFP group (0.51±0.06 vs 0.33±0.03 $bpm.mmHg^{-1}$, respectively, $p<0.05$, Figure 3-3). Interestingly, BRG of WKY LVV-hKir2.1 (1.29±0.18 $bpm.mmHg^{-1}$) was also increased in comparison to WKY LVV-eGFP group (0.41±0.02 $bpm.mmHg^{-1}$, $p<0.0001$), despite all cardiovascular variables remaining unchanged.

RespR remained unchanged throughout the full experimental protocol in all animal groups, before and after the lentiviral microinjection. At 60 days after microinjection, the baseline values of respiratory rate in the anesthetized animal were 76±3.4, 81±4.9, 80±4.5 and 67±3.5 cpm, respectively for SHR and WKY LVV-hKir2.1, SHR and WKY LVV-eGFP. However, peripheral chemoreceptor reflex activation with lobeline, elicited a

hyperventilatory reflex responses of different magnitude according to the animal group. In SHR LVV-hKir2.1 animals showed a decreased ventilatory response when compared with SHR LVV-eGFP (Δ24.4±3.4 vs Δ38.1±4.9 cpm, respectively, $p<0.05$) (Figure 3-3). In contrast, there were no differences in the ventilatory response between WKY LVV-hKir2.1 and WKY-eGFP groups (Δ23.3±5.9 cpm for WKY LVV-hKir2.1; Δ24.8±4.2 cpm for WKY LVV-eGFP). Mean BP responses to chemoreflex activation in SHR LVV-hKir2.1 (from 140±7 to 154±9 mmHg) were depressed compared to SHR LVV-eGFP rats. (179±9 to 193±9 mmHg; $p<0.05$) but HR responses were not different (from 337±23 to 359±12 vs 373±10 to 362±13 bpm, respectively). For the two WKY groups changes in BP and HR to peripheral chemoreflex activation were not different.

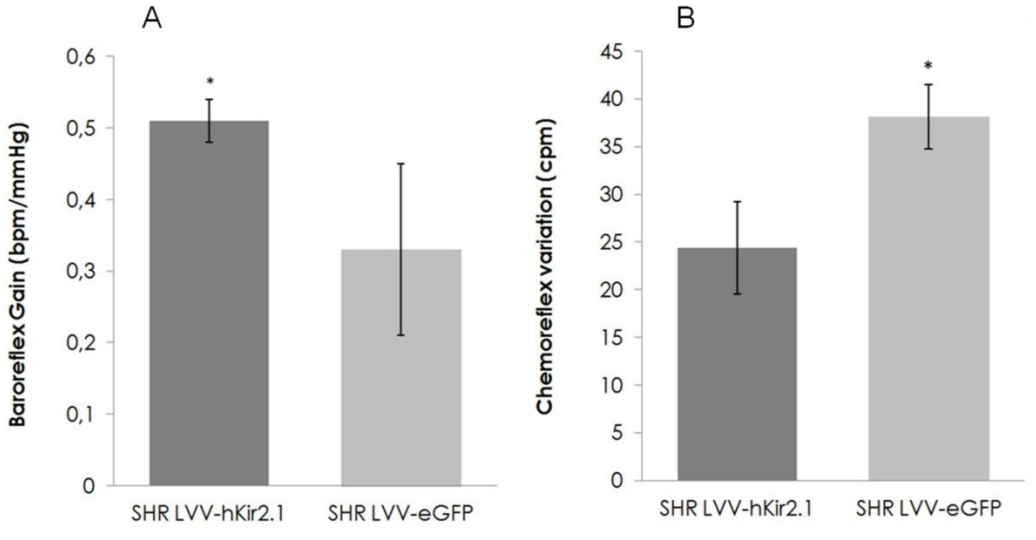


Fig. 3-3 – The histograms show the effect of bilateral microinjections of LVV-hkir2.1 or LVV-eGFP into the PVN on cBRG (A) and chemoreflex variation (B), 60 days pos-microinjection. In SHR-hKir2.1 there is an increase in the baroreflex gain and a decrease in the chemoreflex ventilatory response. The asterisks denote statistically significant differences between groups; *p < 0.05. Abbreviations: cpm, cycles per minute.

3.4. Circadian variation of BP and HR and patterns of nocturnal blood pressure profile

In basal conditions and without any intervention, the pattern of circadian variation of BP and HR followed a similar trend- lower BP values during the light phase relative to the dark phase. During the light phase systolic, diastolic and mean BP of SHRs were

significantly higher than those for WKY rats (Table 3.1; $p<0.0001$) over the same time period. The same type of variation was found for the dark phase where SHRs showed higher values for BP parameters than WKY ($p<0.0001$; Table 3.1). Mean basal HR followed these variations in BP inversely. The HR was significantly lower during the light and dark phases for SHR than for WKY rats ($p<0.01$; Table 3.1).

At 60 days after the LVV-hKir2.1 microinjection, SHRs showed a significant decrease of systolic, diastolic and mean BP during both light and the dark phases (both $p<0.01$; Table 3.1). A significant decrease of HR was observed during the light but not during the dark phase ($p>0.05$). For the SHR LVV-eGFP rats HR, diastolic, systolic and mean BP values for the light phase and dark phase were expectedly increased at 60 days (Table 3.1). Finally, in WKY LVV-hKir2.1 as well as WKY LVV-eGFP rats there was an increase in BP during the dark phase without a distinct circadian rhythm. This profile was maintained after LVV-hKir2.1 and LVV-eGFP PVN microinjections at 60 days (Table 3.1).

Table 3.1 – Blood pressure and heart rate during the light and dark phases for all groups before and 59 days after the microinjection.

	Light phase				Dark phase			
Group	SBP	DBP	MBP	HR	SBP	DBP	MBP	HR
Basal								
SHR	156 ± 3	132 ± 3	140 ± 3	297 ± 6	160 ± 4	137 ± 4	145 ± 4	325 ± 6
WKY	118 ± 3	90 ± 2	100 ± 1	362 ± 9	120 ± 3	92 ± 2	102 ± 2	373 ± 11
59 days after microinjection								
SHR LVV-hKir2.1	131 ± 5*	113 ± 4*	119 ± 4*	271 ± 2	133 ± 7*	114 ± 6*	120 ± 6*	320 ± 5*
SHR LVV-eGFP	172 ± 11	145 ± 11	154 ± 10	264 ± 5	177 ± 10	152 ± 11	160 ± 10	305 ± 7
WKY LVV-hKir2.1	117 ± 4	87 ± 3	97 ± 2	340 ± 10	121 ± 2	91 ± 4	101 ± 3	378 ± 12
WKY LVV-eGFP	114 ± 2	88 ± 3	96 ± 2	354 ± 5	116 ± 2	92 ± 3	100 ± 2	389 ± 2

Values are expressed as means ± SEM. Abbreviations: DBP, diastolic blood pressure; HR, heart rate; MBP, mean blood pressure; and SBP, systolic blood pressure; SHR LVV-hKir2.1, Spontaneously hypertensive rats microinjected with LVV-hKir2.1; SHR LVV-eGFP, Spontaneously hypertensive rats microinjected with LVV-eGFP; WKY LVV-hKir2.1, Wistar Kyoto rats microinjected with LVV-hKir2.1; WKY LVV-eGFP, Wistar Kyoto rats microinjected with LVV-eGFP. *$P < 0.01$, statistically significant difference between basal and day 59 values.

3.5. Metabolic evaluation

A significant decrease in food intake was observed in SHR LVV-hKir2.1 at 60 days after the microinjection (Table 3.2). No other significant changes were found in body weight, water intake, feces and urine production for all groups, before and after the microinjections suggesting that the physical inactivity due to social isolation (only one animal per cage) could have an impact on food consumption. Furthermore, animals were not subjected to

an adaptation period to the metabolic cages, which could impact on our metabolic data, constituting a study limitation.

Table 3.2 – Metabolic evaluation of spontaneously hypertensive rats before and 59 days pos-injection.

Group	ΔWeight (g)	Food (g)	Water (ml)	Faeces (g)	Urine (ml)
Before microinjection; basal conditions					
SHR LVV-eGFP	−1 ± 2.0	19 ± 3.5	27 ± 2.2	9 ± 2.1	11 ± 1
SHR LVV-hkir2.1	−1 ± 1.4	24 ± 1.3	40 ± 4.2	14 ± 3.1	16 ± 3.5
After microinjection (at 59 days)					
SHR LVV-eGFP	−3 ± 1.3	27 ± 1.6	31 ± 4.0	13 ± 1.5	12 ± 0.9
SHR LVV-hkir2.1	−1 ± 0.6	20 ± 0.6*	32 ± 4.9	8 ± 0.9	16 ± 2.1

Values are expressed as means ± SEM. Abbreviations: SHR LVV-hKir2.1, Spontaneously hypertensive rats microinjected with LVV-hKir2.1; SHR LVV-eGFP, Spontaneously hypertensive rats microinjected with LVV-eGFP; WKY LVV-hKir2.1, Wistar Kyoto rats microinjected with LVV-hKir2.1; WKY LVV-eGFP, Wistar Kyoto rats microinjected with LVV-eGFP. *$P < 0.05$, statistically significant difference between basal and day 59 values.

3.6. Histological, immunohistochemical and Western blot analysis

The microinjection sites were located within the PVN according to the rat atlas of Paxinos & Watson (1986). Enhanced green fluorescent protein was detected by fluorescence microscopy as fluorescence confined to a surface of 0.10–0.20mm around the injection site. The eGFP did not penetrate the third ventricular ependymal lining. Through immunohistochemical studies, it was confirmed that PVN neurones expressed eGFP (Fig. 3-4). The overexpression of hKir2.1 in the PVN was analysed using Western blot. The PVN dissected from SHRs microinjected with LVV-hKir2.1 showed an increased expression of hKir2.1, on average about ninefold increased when compared with the LVV-eGFP group (Fig. 3-4).

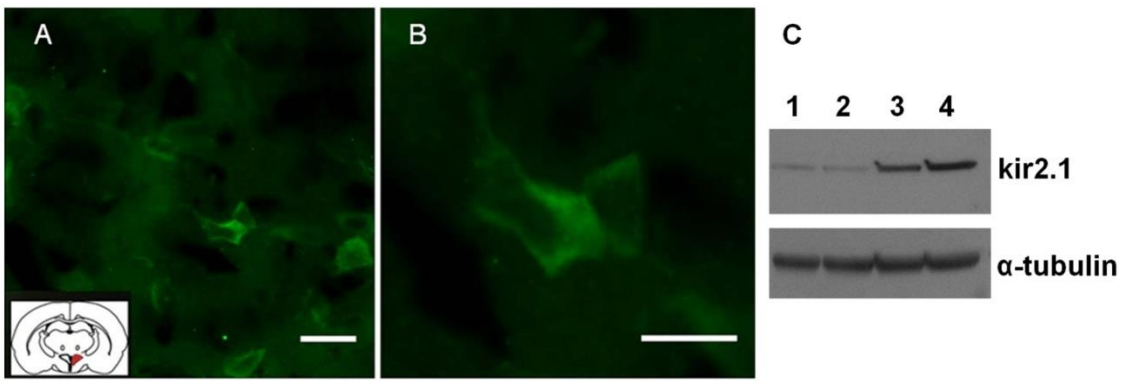


Fig. 3-4 – Lentiviral vector-mediated transduction of green fluorescent protein (GFP) in the paraventricular nucleus (PVN); confocal microscope images of GFP-expressing cells in the PVN (bar: (A)

20 µm, (B) 10 µm) following injection of lentiviral vector into this site. (C) Western blot analysis of sham SHR (1,2) and LVV-hKir2.1 microinjected SHR (3,4). Results show an over expression of hKir2.1 in LVV-hKir2.1 microinjected SHR. α-tubulin was used as house keeping gene.

4. DISCUSSION

In the present study, we investigated the effect of over expressing a potassium inwardly rectifying channel in the PVN to lower neuronal activity while measuring BP chronically and its reflex control in a rat model of hypertension. Our study is the first to demonstrate that chronic suppression of PVN neuronal activity in freely moving SHRs causes a sustained reduction in arterial blood pressure (>60 days) together with a decrease of sympathetic activity, a down-regulation of peripheral chemoreflex responsiveness and an improvement of baroreflex gain. No such changes were found in the control groups of both rat strains that underwent comparable experimental protocols.

The PVN nucleus of the hypothalamus is well known for its importance in autonomic control and, in particular, for cardiovascular regulation. Several anatomical and electrophysiological studies have shown that PVN neurones project either directly to the spinal cord or to the RVLM (Coote, 2007) thereby accessing sympathetic neurones to modulate blood pressure (Hosoya *et al.*, 1991; Loewy, 1991; Coote, 1995; Ranson *et al.*, 1998; Motawei *et al.*, 1999; Pyner & Coote, 1999, 2000; Badoer, 2001; Coote, 2005). As an example, electrolytic lesions of the PVN in SHR elicited an acute reduction of sympathetic activity together with a decrease of blood pressure (Takeda *et al.*, 1991). Other acute studies, performed under general anesthesia, showed that PVN muscimol injections lowered BP and renal sympathetic nerve activity both in SHR and WKY rats, indicating that this region was tonically active in both animal strains to control BP and peripheral sympathetic activity (Allen, 2002).

In the SHR, sympathetic activity is known to be over-activated even before hypertension develops (Simms *et al.*, 2009). Several studies have pointed out that the persistent increase in sympathetic tone is a major contributor to both the initiation and maintenance of the hypertensive condition (Yamada *et al.*, 1988; Grassi, 2004b; Smith *et al.*, 2004; Guyenet, 2006; Fisher & Paton, 2012). In fact, increased sympathetic activity has been detected in normotensive individuals with a family history of hypertension and

in individuals with essential hypertension but not in those with secondary hypertension (Yamada *et al.*, 1988; Grassi *et al.*, 1998; Grassi, 2004a, 2009). Likewise, high plasmatic nor-epinephrine levels have also been associated with essential hypertension being consistently increased in younger hypertensive patients (Grassi, 1998) and increased peripheral sympathetic nervous activity has been detected by microneurography techniques in hypertensive patients (Anderson *et al.*, 1989; Grassi, 1998; Greenwood *et al.*, 1999; Mano, 2012).

Several studies both in human subjects and animal models have demonstrated an association between the circadian variation of BP values, the hypertensive condition, the sympathetic activation, the end-organ damage and the worsening of cardiovascular outcome (White, 2000; Pickering & Kario, 2001; Weber, 2002). Thus, the idea of a long term modulation of the level of sympathetic activity, at its central origin, as a way to control, and treat, high blood pressure and increasing cardiovascular compliance is very appealing. In particular, the manipulation of sympathetic cell excitability by modulation one of K^+ channel expression, to hyperpolarize neuronal resting membrane potential, is an attractive hypothetical therapeutic strategy (Duale *et al.*, 2007).

In the present work, our purpose was to depress chronically the activity of PVN neurones by the over-expression of K^+ channels in PVN neurones exclusively to evaluate its consequences upon long term blood pressure regulation in an animal model of hypertension.

We overexpressed a human inwardly rectifying potassium channel (hKir2.1) under the control of a synapsin promoter that was neurone specific (Duale *et al.*, 2005a; Duale *et al.*, 2005b). Lentivirus was used because its expression has been shown to be sustained within PVN neurones in the long term (Coleman *et al.*, 2003). In previous studies, Duale et al. (2007) and Howorth et al. (2009) showed that hKir2.1 overexpression hyperpolarized the membrane potential of cultured cathecolaminergic PC12 cells by ~10 mV, which is expected to result in 'electrical silencing' of PVN neurones (Duale *et al.*, 2007; Howorth *et al.*, 2009). Similar overexpression strategies have been used to reveal that electrical silencing of neurones affected development in ovo (Yoon et al. 2008), neuronal activity in vivo (Okada & Matsuda, 2008) and the ability of neurons to make and maintain connections in vivo (Yu *et al.*, 2004; Mizuno *et al.*, 2007; Hendy, 2010). This virus

mediated approach has the advantage of being site specific and enabling overexpression in adulthood, which avoids the development of putative compensatory mechanisms associated with transgenic animals (Hendy, 2010).

Our results show that LVV-hKir2.1 treatment of the PVN in SHRs lowered SBP by ∼15% (>20 mmHg). This decline of SBP, which was accompanied by a decrease in HR, was statistically confirmed at 30 days after the lentiviral microinjection and persisted until the animals were killed 60 days postinjection. Interestingly, the LF spectra of SBP (indicative of sympathoinhibition) occurred before the fall in SBP (i.e. 20 versus 30 days), suggesting a putative association between the changes in both variables.

Furthermore, the fall in HF SBP is indicative of reduced respiratory modulation of arterial pressure and could include reduced respiratory–sympathetic coupling, a phenomenon known to raise total peripheral resistance in the SHR (Simms *et al.*, 2009). In contrast, changes in diastolic BP were significant only after 50 days, suggesting the involvement of an additional mechanism. This reveals novel insight into the long-term control of arterial pressure in hypertension by the PVN. It also indicates that the system does not adapt. This could be explained by the associated improvement of baroreflex gain and/or a downregulation of peripheral chemoreflex responsiveness to stabilize lower levels of blood pressure, as we observed. We propose that these changes were a result of reduced electrical excitability of PVN premotor sympathetic neurones, but we cannot rule out reduced release of vasopressin and oxytocin.

This is consistent with our neuroanatomical Western blot analysis confirming that hKir2.1 protein overexpression was within the PVN region. Interestingly, respiratory rate remained unchanged in all experimental groups, suggesting that there is no tonic excitatory drive from the PVN affecting this variable in hypertensive or normotensive rats. Additionally, we saw no tonic influence from the PVN on the resting arterial pressure level in normotensive rats, which contrasts with a previous acute in vivo study (Allen, 2002).

It is well accepted that neurogenic hypertension is accompanied by an impairment of the baroreceptor reflex (Grassi *et al.*, 1998).Our data showed that depressing PVN neuronal activity improved baroreflex gain. Previous work from several authors has shown that during the course of an alerting reaction there is a decrease in baroreflex efficacy and a

facilitation of the carotid chemoreceptor reflex due to modifications of synaptic integration at the level of the nucleus tractus solitarii; this might include mechanisms involving GABA and angiotensin II release within the nucleus tractus solitarii (Jordan *et al.*, 1988; KM, 1990; Silva-Carvalho *et al.*, 1995a; Silva-Carvalho *et al.*, 1995b; Kasparov *et al.*, 1998; Kasparov & Paton, 1999; Head & Mayorov, 2001; Rocha *et al.*, 2003). Such an angiotensinogenic mechanism seems to be particularly active in pathophysiological conditions such asmyocardial ischaemia and hypertension (Rocha *et al.*, 2003; Rosário *et al.*, 2003; Maximino *et al.*, 2006), and its behaviour can be modulated by intervening pharmacologically on AT1 receptors within the nucleus tractus solitarii (Kasparov *et al.*, 1998; Kasparov & Paton, 1999; Rocha *et al.*, 2003; Rosário *et al.*, 2003). In fact, during myocardial ischaemia, AT1 blockade reversed the remodelling of baroreceptor and chemoreceptor reflex function in a way similar to that elicited upon the overexpression of hKir2.1 in PVN neuronal cells (Rocha *et al.*, 2003; Rosário *et al.*, 2003).

The demonstration of a non-dipper blood pressure profile in animal models remains difficult, mainly due to the failure to establish a clear distinction between day and night values. This was confirmed in our study, because through PVN-induced sympathetic manipulations, we were only able to modify BP light–dark values of SHRs which approached those of WKY rats. However, we were unable to modify the day and night profile of BP value variations in both strains. This inability to define a light–dark profile in rats similar to the one set for human subjects may be due to the intermittent behaviour rats, with alternating awake and sleep periods in both the light and the dark phase. It is likely that the only way to define the light and dark phase profiles of rats better would be by monitoring of cerebral activity through EEG, which was outside the scope of the present work.

In conclusion, the present work shows that the intervention on central sympathoexcitatory neurone excitability through the genetic manipulation of expression of K+ channels is able to alter peripheral blood pressure in the long term. This occurs by remodelling of the sympathetic outflow and restores the imbalance of peripheral reflex mechanisms that maintain cardiovascular homeostasis. Our data, from an animal model, give insights into the pathophysiological mechanisms involved in the aetiology of neurogenic hypertension and provide novel hypothetical therapeutic interventions at

both the central and the peripheral level of the autonomic nervous system to control sympatoexcitation.

New findings and their importance under working hypothesis 1

We are able to show, for the first time, that overexpression of an inwardly rectifying potassium channel in the paraventricular nucleus provided a long-term (>60 days) antihypertensive response in conscious spontaneously hypertensive rats that was associated with a reduction in neurohumorally mediated vasoconstriction, enhanced baroreflex sensitivity and reduced peripheral chemosensitivity; no such response was observed in normotensive rats. Our results support the paraventricular nucleus as a therapeutic target for the chronic control of blood pressure in neurogenic hypertension under the concept of autonomic therapeutics.

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Chronic depression of hypothalamic paraventricular neuronal activity produces sustained hypotension in hypertensive rats by V. Geraldes, N Gonçalves-Rosa, B Liu, JF Paton and I Rocha

HYPOTHESIS 2

The excitation of PVN neurons elicits sympathoexcitation and pressor responses through excitatory connections with the rostro ventrolateral medulla. RVLM is a key area of the brainstem that regulates the rate and pattern of discharge of sympathetic pre-ganglionic neurons which are the major determinants of the sympathetic output to the heart and vessels as well as to the kidney. Despite receiving inputs from several central autonomic nuclei, a direct angiotensinogenic pathway from PVN to RVLM involved in cardiovascular reflexes regulation was described. Sympathetically mediated pressor responses have also been evoked from the medullo-cervical pressor area, an area that extends till the third cervical segment. These responses are not relayed through rostroventrolateral medulla and can be elicited when the brainstem is transacted. MCPA cells project to spinal neurons that directly innervate sympathetic preganglionic neurons. In accordance, the following working hypothesis was built:

What is the role of rostral ventrolateral medullary activity in the long term maintenance of high blood pressure values, sympathoexcitation and baroreflex blunting?

1. INTRODUCTION

Hyperactivity of the sympathetic nervous system has for a long time been hypothesized as a mechanism for the initiation, development and maintenance of elevated blood pressure (BP) in human hypertensive patients and animal models (Bourjeili *et al.*, 1995; Esler, 1995; Johansson *et al.*, 1999; Mancia *et al.*, 1999; Carlson *et al.*, 2000; Grassi, 2004b; Fisher & Paton, 2012). Recently, the development of non-pharmacological therapeutics through medical devices for the treatment of resistant hypertension has further emphasized the association between sympathetic hyperactivity and the generation of high blood pressure. The findings that baroreflex impairment has been associated with a higher risk of developing hypertension in normotensive children with a family history of hypertension (Yamada *et al.*, 1988) suggests a neurogenic component could be causal. This interpretation is consistent with increased levels of sympathetic activity and plasma noradrenaline in white coat and borderline hypertensive individuals

(Grassi, 1998, 2004a; Smith *et al.*, 2004; Grassi, 2009). These studies imply that sympatho-excitation precedes hypertension and may be a cause for this condition (Lucini *et al.*, 2002; Guyenet, 2006). The increase of sympathetic drive to the heart and peripheral circulation not only increases cardiac output and vascular resistance, causing elevated BP values (Schlaich *et al.*, 2012), but also to end organ damage (Zubcevic *et al.*, 2011), which worsens patient prognosis.

Based mainly on anaesthetized animals, the rostroventrolateral medulla (RVLM) has been shown to be a pivotal area regulating cardiovascular sympathetic tone. The RVLM lies ventral to the rostral part of the nucleus ambiguous (NA), caudal to the facial nucleus and ventral to the Bötzinger complex (Dampney, 1994; Janig, 2006b). The RVLM neurones project to the sympathetic preganglionic neurones in the intermediolateral (IML) cell column of the spinal cord (Guertzenstein & Silver, 1974; Dampney, 1994; Leman *et al.*, 2000; Card *et al.*, 2006b) and receives a direct glutamatergic projection from the NTS, believed to be part of the peripheral chemoreflex (Ross *et al.*, 1985; Koshiya & Guyenet, 1996b; Nosjean *et al.*, 1998). There are also projections from the PVN to the RVLM (Kantzides & Badoer, 2005; Pyner, 2009). Functionally, electrical or chemical activation of RVLM evokes a pressor response which is blocked by adrenoreceptor antagonists (Ross *et al.*, 1984; Kuo & Yang, 2000). In contrast, inhibition of the RVLM caused a decrease in BP, HR and sympathetic nerve activity in the conscious normotensive rat, from day 5 to day 10 (Dampney, 1994; Kishi *et al.*, 2001). In acute studies, in chloralose-anesthetized SHR, bilateral injection of excitatory amino acid antagonist kynurenic acid (KYN) into the RVLM reduced mean arterial pressure by ≈40 mmHg, however in WKY similar injections did not alter BP (Ito *et al.*, 2000).

Overexpression of MnSOD (Kishi *et al.*, 2004; Nishihara *et al.*, 2012), or microinjection of tempol (Kishi *et al.*, 2004; Koga *et al.*, 2008; Konno *et al.*, 2012) decreased blood pressure, heart rate (HR) and urinary norepinephrine excretion in stroke-prone spontaneously hypertensive rats, but not in normotensive rats. Furthermore, other studies demonstrated that overexpression of inducible NO synthase (iNOS) in the RVLM elicited blood pressure elevation and sympathoexcitation in normotensive rats via increase in oxidative stress (Kimura *et al.*, 2005) and a significant reduction in the molecular synthesis

and functional expression of iNOS in the RVLM produces the opposite effects (Chan *et al.*, 2001a; Chan *et al.*, 2001b).

In chloralose-anesthetized SHR, bilateral injection of excitatory amino acid antagonist kynurenic acid (KYN) into the RVLM reduced mean arterial pressure by ≈40 mmHg, however in WKY similar injections did not alter BP (Ito *et al.*, 2000).

It is under powerful baroreceptor reflex control, which provides profound inhibition to these neurons (Cravo *et al.*, 1991; McAllen & May, 1994; Lipski *et al.*, 1996; Guyenet, 2006). This inhibition originates from GABA-ergic neurons located within the caudal ventrolateral medulla (CVLM) that are driven by glutamatergic neurons from the nucleus tractus solitarius (NTS) receiving baroreceptor inputs (Guyenet, 2006; Janig, 2006a). Bilateral lesions of the RVLM in anaesthetised animals results in a decrease in BP to levels comparable to those seen after spinal cord transection (Guertzenstein & Silver, 1974; Dampney & Moon, 1980). Less is known about the role of the RVLM after chronic lesions in conscious rats.

In the hypertensive state the role of RVLM neurons in the regulation of SNA and BP in conscious animals is not completely understood. To date, most of our knowledge of the RVLM for BP control in hypertension is restricted to anaesthetized animals. The central processing of baroreceptor reflexes in spontaneously hypertensive rats (SHR) appears normal, but CVLM-mediated inhibition of the RVLM sympathoexcitatory neurons seems to be attenuated, suggesting that it is specifically a baroreceptor-independent mechanism of cardiovascular regulation in SHR that is altered (Sved *et al.*, 2000). It has also been reported that RVLM neurons in SHR are over-activated and possibly this mechanism may lead to increased BP and peripheral sympathetic nerve activity (Matsuura *et al.*, 2002). The recent work of Moraes *et al*. indicates that the firing frequency of RVLM neurones, through there respiratory modulation, is exacerbated in the SHR (Moraes *et al.*, 2014). Our aim was to understand the importance of the RVLM for the maintenance of hypertension in conscious unrestrained adult SHR. Given the inevitable compensation that follows electrolytic and chemically induced lesions, we have used a virus to express a potassium channel to depress the electrically excitability of neurons. In previous studies, we have shown that lentiviral vectors (LVV) over expression of inward rectifying potassium channels hKir2.1 depressed the electrical excitability of NTS neurons (Duale *et*

al., 2005b; H *et al.*, 2005). More recently, we showed a long term decrease in arterial pressure and sympathetic activity following LVV over expression of hKir2.1 channels in the paraventricular nucleus of the hypothalamus (Geraldes et al., 2013). Using this approach we have studied the chronic effect of depressing the intrinsic excitability of RVLM neurons on blood pressure and sympathetic activity in SHR. We have compared the importance of the RVLM with another more recently described sympathoexcitatory region – the medullo-cervical pressor area (MCPA). This pressor and sympathoexcitatory region is not dependent on the integrity of the RVLM (Seyedabadi et al., 2006). It extends caudally from the medulla at the level of the caudal pole of the inferior olive to the fourth cervical segment and contains spinally projecting neurons (which are neurochemically heterogeneous) that directly innervate the sympathetic preganglionic neurons (Seyedabadi *et al.*, 2006). It is distinct from the caudal pressor area, because blockade of the RVLM with muscimol inhibited this pressor response but not that evoked from the MCPA (Seyedabadi et al., 2006). The role of this novel descending sympathoexcitatory region in central cardiovascular regulation remains to be elucidated.

Here we show that expression of hKV1.2 channels in RVLM neurons causes a long lasting reduction in arterial pressure in SH rats but that no such chronic response was obtained from the MCPA.

2. MATERIALS AND METHODS

All the experimental procedures were in accordance with the European and Portuguese Law on animal welfare and had the approval of the ethic committee of the Faculty of Medicine, University of Lisbon, Portugal. Spontaneous Hypertensive Rats (SHR), males, aged 12 weeks and weighing 351±10g, were from Charles River Laboratory. Animals, synchronized for a 12:12h light-dark cycle (light on at 7am, light off at 7pm), were housed individually and allowed to freely move in standard plastic cages in a climate-controlled room (22±1ºC). Food and water were provided *ad libitum*.

2.1 Animal model of hypertension

We have used the SHR that is a well established model of hypertension characterized by increased plasma catecholamine levels (Nagaoka & Lovenberg, 1976), increased sympathetic nerve activity (Judy *et al.*, 1976), sympathetic hyperreactivity to stressful stimuli (Lundin & Thorén, 1982) and a faster firing rate of RVLM neurons (Chan *et al.*, 1991; Matsuura *et al.*, 2002; Matsuura *et al.*, 2005) when compared with its normotensive control, the Wistar-Kyoto rat (WKY).

2.2. Viral vector construction and validation

Lentiviral vector (LVV) construction was based on previous studies (Waki *et al.*, 2003; Duale *et al.*, 2007; Geraldes *et al.*, 2013). The LVV-eGFP, used for the sham group, was a mix of LVV-TREtight-GFP 5.7x10E9 and LVV-Syn-Eff-G4BS-Syn-Tetoff 6.2x10E9 in a ratio 1:4. These binary systems express enhanced Green Fluorescent Protein (eGFP). The LVV-hKir2.1 is mix of LVV-TREtight-Kir-cIRES-GFP 5.4x10E9 and LVV-Syn-Eff-G4BS-Syn-Tetoff 6.2x10E9 in a ratio 1:4, that expresses eGFP and human inwardly rectifying potassium channels (hKir2.1) in neurones. Validation of transduction efficacy and transgene expression was assessed as described previously by us (Duale *et al.*, 2007; Geraldes *et al.*, 2013) and included mRNA expression, immunocytochemical and electrophysiological data.

2.3. Microinjection sites

Initially, we fine-tuned our stereotaxic coordinates for bilateral RVLM and MCPA microinjections in 10 SHR anaesthetised with sodium pentobarbitone (60mg/Kg, IP). Bilateral microinjections (0.05µl) of LVV-eGFP were performed. Using fluorescence microscopy and histological reconstruction, we determined the correct coordinates for RVLM and MCPA microinjections and the amount of LVV-eGFP needed to limit transduction to the confines of the RVLM and MCPA.

2.4. Surgery

SHR were divided into 4 groups according to the region and microinjection content: RVLM LVV-hKir2.1 (n=6), MCPA LVV-hKir2.1 (n=6) and RVLM LVV-eGFP (n=6), MCPA LVV-eGFP (n=5).

a) Implantation of radio-telemetry probes

SHR were anaesthetised (sodium pentobarbitone, 60mg/kg, i.p.) and a medial laparotomy was performed to allow the insertion of the telemetry sensor catheter (ca. 0.7 mm, thin-walled thermoplastic membrane) into the root of the abdominal aorta, below the renal artery, using a binocular microscope. Before implantation, the aorta was clamped proximally and the catheter was inserted and secured with medical glue (Vetbond 3M, Saint Paul, MN, USA). Radiotelemetric pressure transducers (Data Sciences International, St. Paul, Minnesota, MN, USA) consisting of a fluid-filled catheter connected to a PA-C40 transmitter was sutured in the abdominal wall to allow the transmission of blood pressure (BP) values over months. Antibiotics (Baytril, 5 mg/kg) and analgesics (Rimadyl, 4 mg/kg) were subcutaneous injected at the end of the surgery. The animals were allowed to recover for two weeks before viral gene transfer and blood pressure (BP) and heart rate (HR) were continuously monitored by telemetry.

b) Bilateral microinjection into RVLM and MCPA

Two weeks after the probes were implanted, SHR rats were placed in a stereotactic frame (Kopf Instruments) and a craniotomy performed using our previously determined coordinates for LVV-hKir2.1 microinjections (0.05µl) into the RVLM (B: -12.5mm, L: 2.1mm, D: 8mm) or MCPA (B: -14,8mm, L: 2mm, D: 4,5mm)(Paxinos & Watson, 1986). Sham rats were microinjected in the same region with LVV-eGFP. All microinjections were performed bilaterally. Animals of all groups were allowed to recover and monitored by telemetry for 60 days. Heart rate (HR) and blood pressure (BP; systolic, diastolic and mean) were recorded continuously.

2.5. Metabolic Evaluation

Rats were housed individually for 24h in metabolic cages to evaluate food and fluid intake, urine and faeces production and body weight, before and 59 days after the microinjections.

2.6. Cardio-respiratory reflexes evaluation

At 60 days, the SHR were anesthetised (sodium pentobarbitone, 60mg/Kg, IP). The trachea was cannulated below the larynx to record tracheal pressure (TP). The femoral and carotid artery (for arterial pressure monitoring) and femoral vein were cannulated. Rectal temperature was maintained at 38±1°C by a servo-controlled heating blanket. The electrocardiogram (ECG) was recorded with the use of needle electrodes inserted into the limbs and HR is derived from the ECG. The respiratory rate (RespR) was obtained though the TP recording. Baroreceptor and peripheral chemoreceptor reflexes were activated twice with an interval of 5 minutes between each stimulation. The baroreceptor reflex was stimulated using phenylephrine (0.2ml, 25µg/ml i.v.; Sigma Aldrich). Peripheral chemoreceptor reflex was stimulated with lobeline (0.2ml, 25µg/ml, Sigma Aldrich) injected retrogradely via the external carotid artery into the bifurcation of the common carotid artery. HR, BP (systolic, diastolic and mean) and RespR were recorded continuously thought the experiment.

2.7. Histology and immunochemistry

Animals were terminally anesthetized with an overdose of sodium pentobarbitone (60mg/Kg, iv) and immediately perfused transcardially with phosphate-buffered saline (PBS; 0.1M; pH 7.4) followed by 4% paraformaldehyde (0.1M; pH 7.4). The brain was removed and placed for 48 h in 15% (w/v) sucrose solution. Coronal sections (18 µm) were cut on a microtome and mounted on slides. The pipette tip location and the microinjection diffusion in the RVLM were examined and documented. The microinjected contents (LVV-hKir2.1 or LVV-eGFP) containing e-GFP allowed an estimation of virus dispersion. eGFP-labeled fluorescent regions were identified using an epifluorescence

microscope and plotted on standardized sections from the Paxinos and Watson atlas (Paxinos & Watson, 1986).

2.8. Western blot analysis

The expression of hKir2.1 protein in RVLM and MCPA was analysed by western blot following 60 days after the microinjection of LVV-hKir2.1 (n=6) and LVV-eGFP in SHR (n=5). The RVLM and MCPA were dissected from both groups and homogenized by sonication in ice cold RIPA buffer (Sigma) supplemented with a cocktail of protease inhibitors (complete mini, Roche). Proteins were extracted from the homogenates by centrifugation at 5000g for 10 minutes at 4ºC and protein concentration was determined with Bio-Rad DC Protein Assay kit. Proteins were resolved by electrophoresis on a 10% Tris-Glycine SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were blocked with 5% milk in Tween/Tris Buffered Saline (TBST) and incubated overnight at 4ºC with rabbit anti-hKir2.1 polyclonal antibody (Abcam). After washing, membranes were incubated for 1 hour at room temperature with Goat anti-rabbit HRP conjugated (Bio-Rad) and immunoreactive proteins were detected by Immobilon Western Chemiluminescent HRP Substrate (Millipore) and visualized using Curix 60 (AGFA). Membranes were stripped with 0.1M Glycine pH2.2 and reprobed with the α-tubulin antibody (Santa Cruz Biotechnology) for loading control.

2.9. Analysis of BP and HR variability

Telemetric pulsatile blood pressure data were acquired continuously at 1KHz and analyzed with suitable software (LabChart6, Powerlab, ADInstruments). Mean values of HR, BP (systolic, diastolic and mean) and RespR were extracted.

a) Baroreceptor and chemoreceptor reflex

The baroreceptor reflex gain (BRG) was quantified calculating ΔHR/ΔBP ($bpm.mmHg^{-1}$). Chemoreceptor (ChR) reflex was calculated through the RespR derived from the tracheal

pressure before and after stimulation with lobeline: $\Delta ChR = RespR_{lobeline} - RespR_{basal}$. BP and HR were also evaluated.

b) Analysis of BP and HR variability

Systolic BP and RR interval data were analyzed (period of 3 minutes) in the frequency domain (Fast Fourier Transform, FFT), using the in-house software Fisiosinal (Tavares, 2011a), to evaluate sympathetic (Low Frequency band, LF, 0.15-0.6Hz of SBP) and parasympathetic (High Frequency band, HF, 0.6-2.0Hz of HR) activity over time (M Malik, 1996; Marques-Neves *et al.*, 2004).

c) Circadian light/dark heart rate and blood pressure profile

Mean BP and HR values were calculated using the continuous telemetric data and compared between light (7am-7pm) and dark phases (7pm-7am).

2.10. Statistical analysis

Comparisons between groups for the same period and also comparisons within the same group, before and after the microinjections were performed. For the statistical analysis, Student's t test for paired data and ANOVA (with Tukey's range test used as post hoc test) for comparisons between inter-groups were used. All data were expressed as mean±SEM and passed the normality test. Significance was taken as $P < 0.05$.

3. RESULTS

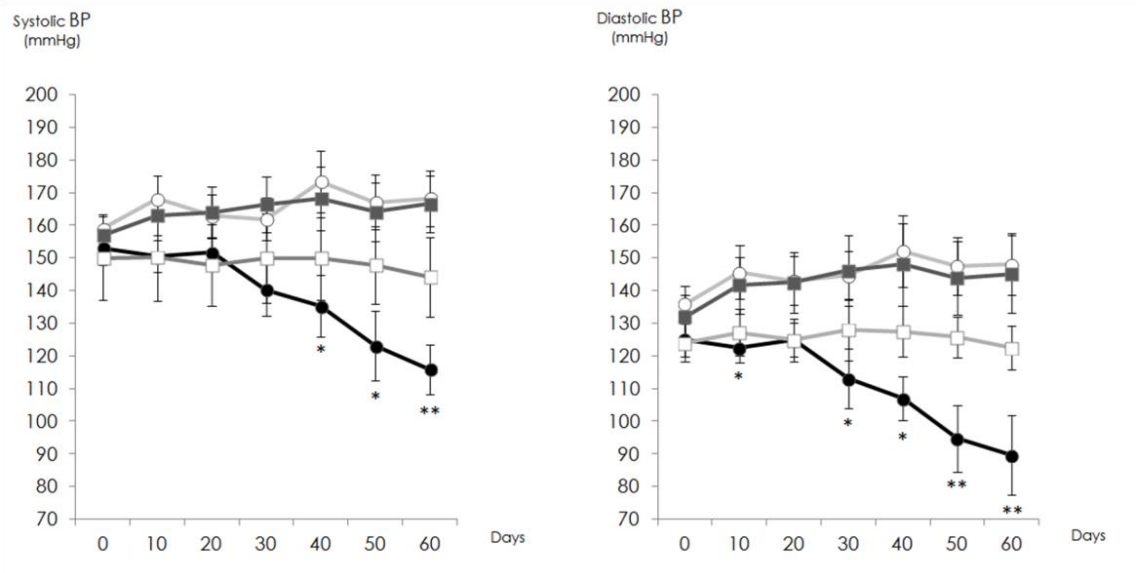
3.1 Effect of LVV-hKir2.1 or LVV-eGFP microinjection on 24h mean values of blood pressure, heart rate and respiration

Basal BP values in conscious SHR (n=23) were 155±3 mmHg (systolic), 130±3 mmHg (diastolic) and mean BP was 138±3 mmHg. HR was 310±4 bpm (Table 3.3). There was a continuous downward trend in BP after RVLM microinjection of LVV-hKir2.1 in with a significant decrease ($p<0.05$) by day 30. To evaluate its persistence, animals were

monitored for a further 30 days. At 60 days post microinjection of LVV-hKir2.1 values for systolic, diastolic and mean BP were 116±8 mmHg, 90±12 mmHg and 98±10 mmHg, respectively. This corresponded to decreases of 39 mmHg, 40 mmHg and 40 mmHg in systolic, diastolic and mean BP, respectively ($p<0.0001$, Figures 3-5). These BP changes in the RVLM SHR LVV-hKir2.1 group were accompanied by a lowering of HR to 293±6 bpm ($p>0.05$), but RespR remained unchanged at all time points. At 60 days after microinjection, the RespR values for RVLM LVV-hKir2.1 group was 72±3 cpm ($p>0.05$).

The RVLM SHR LVV-eGFP group showed increased values of systolic (168±9 mmHg, $p>0.05$), diastolic (148±9 mmHg, $p>0.05$) and mean BP (155±9 mmHg, $p>0.05$) together with a decrease in HR (292±4 bpm, $p>0.05$, Figure 3-5). This profile of BP and HR changes was expected and consistent with their developmental trend (Dickhout & Lee, 1998) .

In the MCPA group, at the 60th day after lentiviral microinjection, BP and HR in SHR LVV-hKir2.1 remained unchanged (Table 3.3, Figure 3-5). Also, RespR didn´t change between the two groups, the RespR values for MCPA SHR LVV-hKir2.1 was 63±3 cpm and for MCPA SHR LVV-eGFP was 63±3 cpm ($p>0.05$).



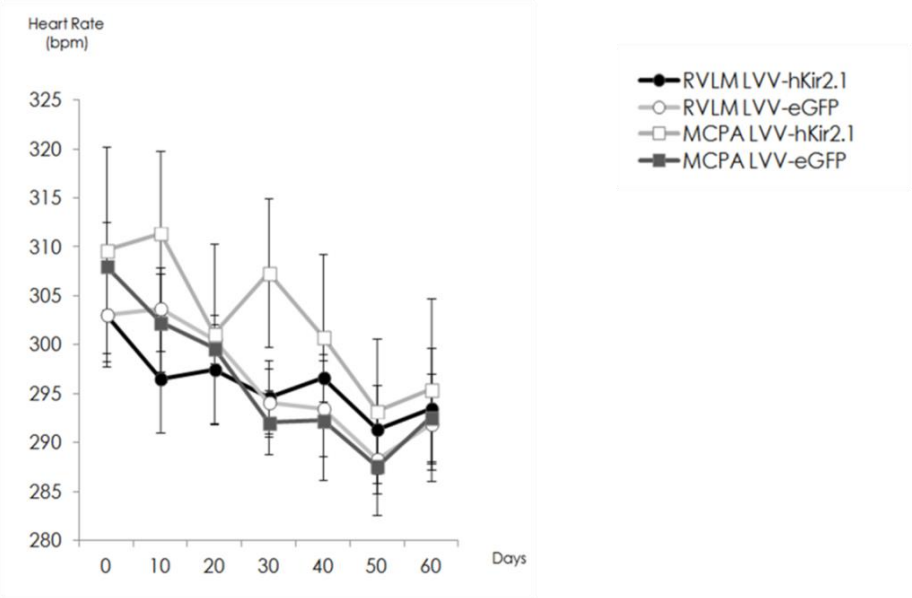


Fig. 3-5 – Effect on systolic, diastolic blood pressure and heart rate in SHR before (0 days) and after microinjection of LVV-hKir2.1 in RVLM (n=6) and in MCPA (n=6) or LVV-eGFP in RVLM (n=6) and in MCPA (n=5). The asterisks denote statistically significant differences between LVV-hKir2.1 and LVV-eGFP groups; *p < 0.05; **p < 0.01.

3.2. Effect of LVV-hKir2.1 microinjection on sympathetic output measured indirectly

SHR showed an overall decrease of cardiovascular autonomic outflow at 60 days after LVV-hKir2.1 microinjection in the RVLM when compared with basal autonomic output at day 0. By using FFT applied to systolic BP and interpulse intervals, a decrease in sympathetic output expressed by LF_{sBP} band power was observed (from 0.69±0.11 to 0.42±0.10 mmHg2, p<0.05). The LF_{sBP}/HF_{RR} ratio for RVLM hKir2.1 was from 0.09±0.02 at the baseline to 0.05±0.01 mmHg2.ms^{-2} at 60 days; p>0.05. In contrast, the LF_{sBP}/HF_{RR} ratio for RVLM LVV-eGFP (from 0.08±0.02 to 0.09±0.03, p>0.05) and the LF (from 0.74±0.13 to 0.86±0.16,p>0.05) remain unchanged. The LF_{sBP} and LF_{sBP}/HF_{RR} ratio was unchanged in MCPA LVV-hKir2.1 (from 1.02±0.23 to 1.03 ± 0.23 mmHg^2 and from 0.067±0.03 to 0.05±0.02 mmHg^2.ms^{-2}; p>0.05). Also in MCPA LVV-eGFP the LF/HF ratio (from 0.099±0.02 to 0.10±0.03 mmHg^2.ms^{-2}; p>0.05) and the LF (from 1.12±0.10 to0.90 ± 0.18 mmHg^2; p>0.05) remain unchanged. The variations of mean LF_{sBP} and LF_{sBP}/HF_{RR}, at 10-days intervals for each SHR group, are depicted in figure 3-6.

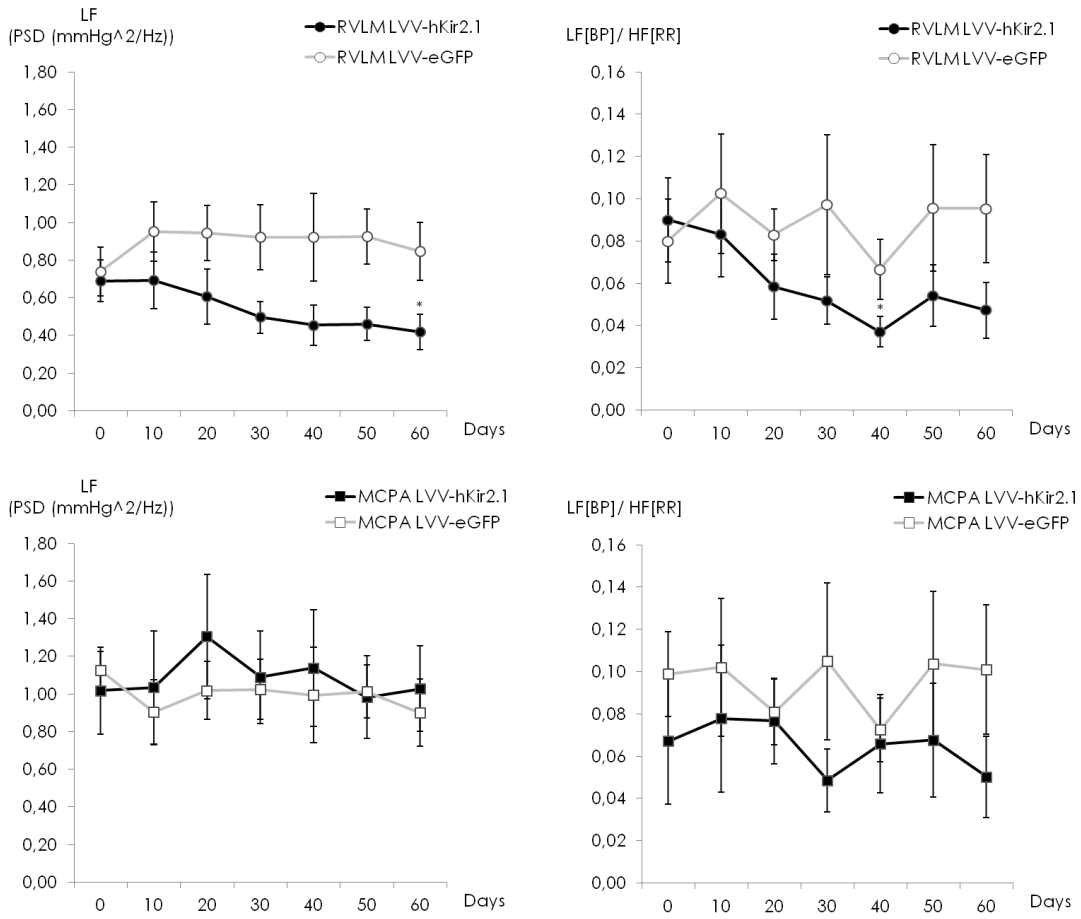


Fig. 3-6 – Mean (±SEM) LF and LF(BP)/HF(RR) before (0 days) and 10 days intervals after the microinjection of LVV-hKir2.1 or LVV-eGFP in RVLM (above) and in MCPA (below). The asterisks denote statistically significant differences between groups; *p < 0.05.

3.3. Arterial baroreflex gain (BRG) and peripheral chemoreflex responsiveness

Injection of phenylephrine (PHE) triggered a progressive increase in mean BP, which was accompanied by a progressive reduction in HR. No changes in the BRG were found in all groups evaluated: in RVLM LVV-hKir2.1 was 0.45±0.07 bpm.mmHg^{-1}, in RVLM LVV-eGFP group was 0.42±0.05 bpm.mmHg^{-1}, in MCPA LVV-hKir2.1 was 0.40±0.05 bpm.mmHg^{-1} and in MCPA LVV-eGFP group was 0.36±0.06 bpm.mmHg^{-1}, p>0.05, Figure 3-7). In RVLM the BP changes to PHE injection in SHR LVV-hKir2.1 was 52±4 mmHg and in SHR LVV-eGFP was 62±5 mmHg (p>0.05). In SHR LVV-hKir2.1 MCPA was 82±7 mmHg and in SHR LVV-

eGFP MCPA was 67±6 mmHg (p>0.05). Between the SHR LVV-hKir2.1 RVLM and SHR LVV-hKir2.1 MCPA groups there were extremely significant differences in the pressor response to PHE injection (p<0.0001).

Peripheral chemoreceptor reflex activation with lobeline, elicited a hyperventilatory reflex responses of different magnitude according to the animal group. In RVLM SHR LVV-hKir2.1 animals the ventilatory response remained unchanged when compared with RVLM SHR LVV-eGFP (Δ24.8±3.0 vs Δ28.9±3.9 cpm, respectively, p>0.05). The same happened to MCPA SHR LVV-hKir2.1 group when compared with MCPA SHR LVV-eGFP (Δ37.7±5.0 vs Δ29.0±4.2 cpm, respectively, p>0.05) (Figure 3-7). Pressor responses to chemoreflex activation in RVLM SHR LVV-hKir2.1 (from 157±8 to 173±10 mmHg) were similar compared to RVLM SHR LVV-eGFP rats (192±9 to 211±9 mmHg; p>0.05) and also HR responses were not different (from 318±14 to 317±16 vs 372±12 to 371±17 bpm, respectively). In MCPA the mean BP and HR responses to chemoreflex activation were unchanged in SHR LVV-hKir2.1 (from 146±9 to 164±7 mmHg and from 341±11 to 343±10 bpm, p>0.05) and in SHR LVV-eGFP (from 178±8 to 195±6 mmHg and from 352±13 to 347±3 bpm).

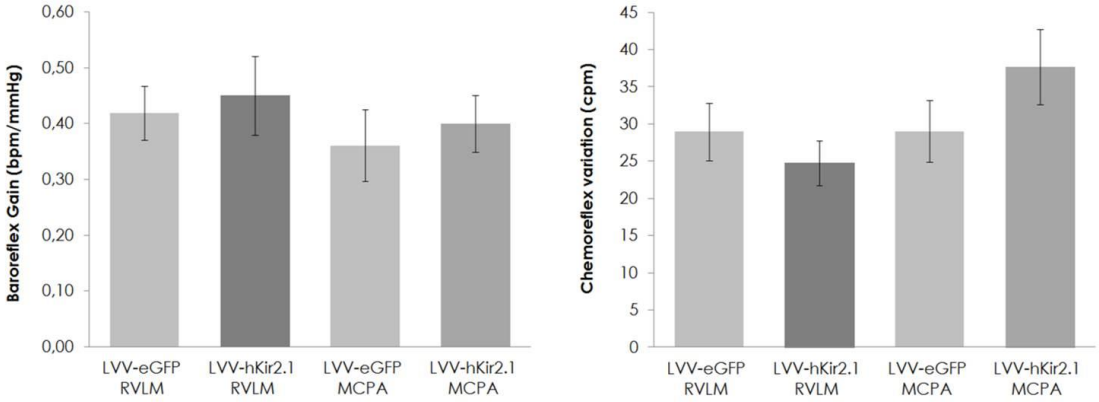


Fig. 3-7 – The histograms show the effect of bilateral microinjections of LVV-hkir2.1 or LVV-eGFP into the RVLM or MCPA on cBRG and chemoreflex variation, 60 days post-microinjection. Abbreviations: cpm, cycles per minute.

3.4. Circadian variation of BP and HR

In basal conditions and without any intervention, the pattern of circadian variation of BP and HR followed a similar trend- lower BP values during the light phase relative to the

dark phase and there were no significant changes between all groups evaluated (Figure 3-8). At 60 days after the LVV-hKir2.1 microinjection, RVLM SHR showed a significant decrease of systolic, diastolic and mean BP during both light and the dark phases (both $p<0.001$; Table 3.3). A significant decrease of HR was observed during the light ($p<0.01$) but not during the dark phase ($p>0.05$). For the RVLM SHR LVV-eGFP rats HR, diastolic, systolic and mean BP values for the light phase and dark phase were expectedly increased at 60 days (Table 3.3). There were no changes in BP values in MCPA SHR LVV-hKir2.1 group (Table 3.3). In MCPA SHR LVV-eGFP rats there was an increase in BP during the both phases (Table 3.3). HR didn´t change in both MCPA groups (table 3.3).

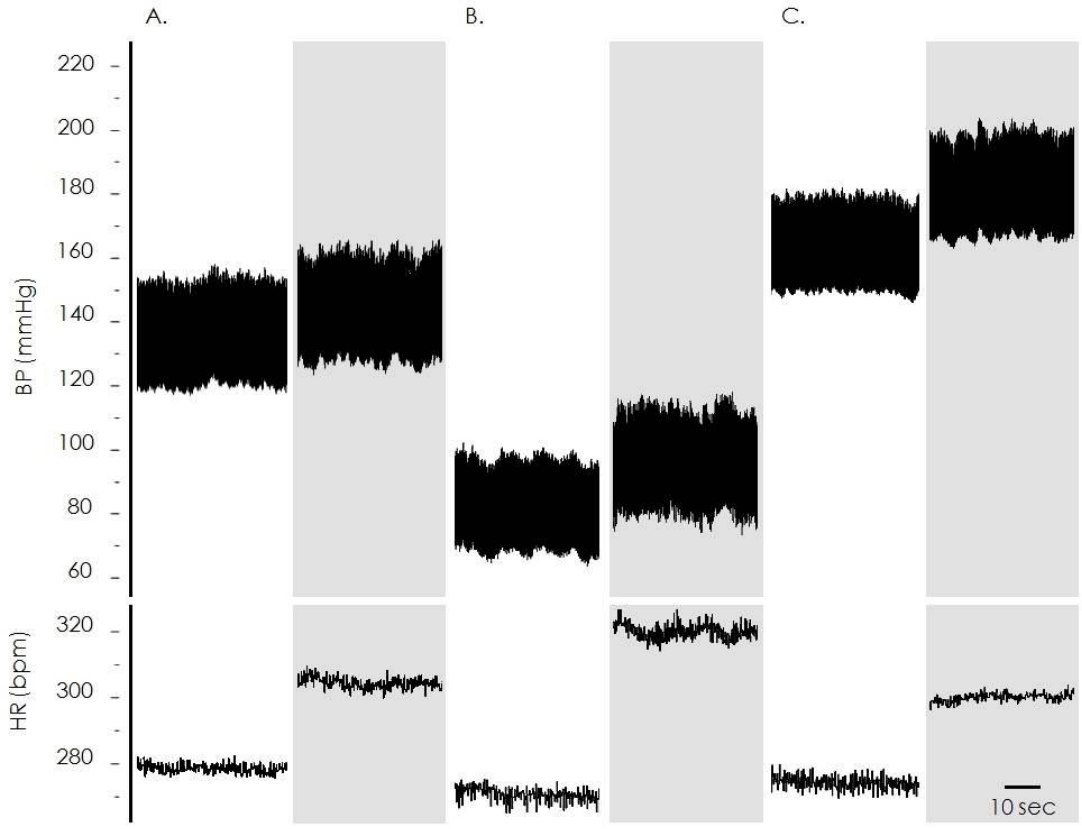


Fig. 3-8 – Raw data showing blood pressure and heart rate: (A) SHR before and (B) 60 days after microinjection of LVV-hKir2.1; (C) another SHR at 60 days after microinjection of LVV-eGFP in RVLM during light (white) and dark (gray) phases.

Table 3.3 – Blood pressure (sBP: systolic Blood Pressure, dBP: diastolic Blood Pressure and mBP: mean Blood Pressure; mmHg) and Heart Rate (HR; bpm) during the light and dark phases for all SHR groups before and 59 days after the microinjection. Values are expressed as mean±SEM. The asterisks denote statistically significant differences between basal and day 59; [a]p<0.05; [b]p<0.01; [c]p<0.001.

	Basal							
	Light phase				**Dark phase**			
	sBP	**dBP**	**mBP**	**HR**	**sBP**	**dBP**	**mBP**	**HR**
RVLM LVV-hKir2.1	152±5	123±5	133±4	284±4	154±5	127±6	136±5	322±6
RVLM LVV-eGFP	158±4	134±5	142±5	290±6	160±4	138±6	146±5	315±6
MCPA LVV-hKir2.1	147±12	121±5	130±7	305±10	153±13	127±6	136±9	354±11
MCPA LVV-eGFP	156±6	131±6	139±6	298±3	158±6	133±7	142±6	318±6

	59 days after microinjection							
	Light phase				**Dark phase**			
	sBP	**dBP**	**mBP**	**HR**	**sBP**	**dBP**	**mBP**	**HR**
RVLM LVV-hKir2.1	115±7[c]	88±12[c]	97±10[c]	267±3[b]	117±8[c]	92±12[c]	100±11[c]	320±10
RVLM LVV-eGFP	166±9	145±10	152±9	272±4[a]	170±8	150±10	157±9	312±4
MCPA LVV-hKir2.1	141±12	120±6	127±8	295±12	147±13	125±7	132±9	336±7
MCPA LVV-eGFP	166±9	144±12	151±11	274±4	167±9	146±12	153±11	311±5

3.5. Metabolic evaluation

No significant changes were found in body weight, food and water intake or in feces and urine production in all four groups before and after microinjections (Table 3.4). Body weights of all groups of animals (hKir2.1 and eGFP) were not significantly different before LVV microinjections. However, all increased at the end of the experience.

Table 3.4 – Metabolic evaluation of SHR before and 59 days pos-injection in RVLM. Values are expressed as mean±SEM. The asterisks denote statistically significant differences between basal and day 59; *p < 0.05.

	Before microinjection – Basal condition				
	ΔWeight (g)	**Food (g)**	**Water(mL)**	**Faeces (g)**	**Urine (mL)**
SHR LVV-eGFP	5.0±6.2	29.0±4.3	30.0±5.0	9.7±3.1	10.3±3.1
SHR LVV-hkir2.1	0.14±5.9	25.6±2.1	41.6±11.8	17.0±5.0	17.1±9.2

	After microinjection (60dpi)				
	ΔWeight (g)	**Food (g)**	**Water(mL)**	**Faeces (g)**	**Urine (mL)**
SHR LVV-eGFP	0.3±2.1	27.0±2.0	32.7±13.7	16.0±3.5	12.3±3.8
SHR LVV-hkir2.1	-4.1±2.1	22.1±3.6	37.9±10.7	13.3±4.1	14.6±3.5

3.6. Immunohistochemical and Western blot analysis

The microinjection sites were located within the RVLM and MCPA according to Paxinos and Watson rat atlas (Paxinos & Watson, 1986). e-GFP was detected by fluorescence microscopy being fluorescence confined to a surface of 0.10 to 0.20 mm around the injection site (Figures 3-9 and 3-10). Through immunohistochemical studies it was confirmed that RVLM and MCPA neurones expressed eGFP (Figures 3-9 and 3-10). The over expression of hKir2.1 in RVLM and MCPA was analyzed using western blot. The RVLM and the MCPA dissected from SHR microinjected with LVV-hKir2.1 showed an increased expression of hkir2.1, on average about 3 times increased in RVLM and 2 times increased in MCPA when compared to LVV-eGFP groups (Figure 3-11).

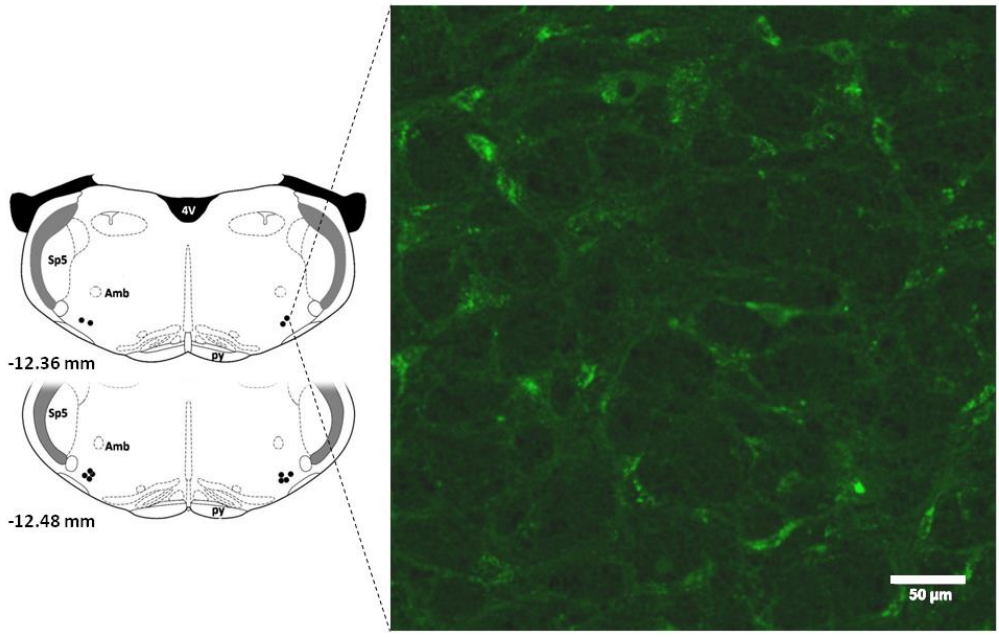


Fig. 3-9 – Localization of the RVLM microinjection sites (black circles) and lentiviral vector-mediated transduction of green fluorescent protein (GFP) in the RVLM; confocal microscope images of GFP-expressing cells in the RVLM *(bar: 50 µm)* following injection of lentiviral vector into this site. Amb, nucleus ambiguous; Py, pyramidal tract; Sp5, spinal trigemina nucleus; 4V, 4th ventricle.

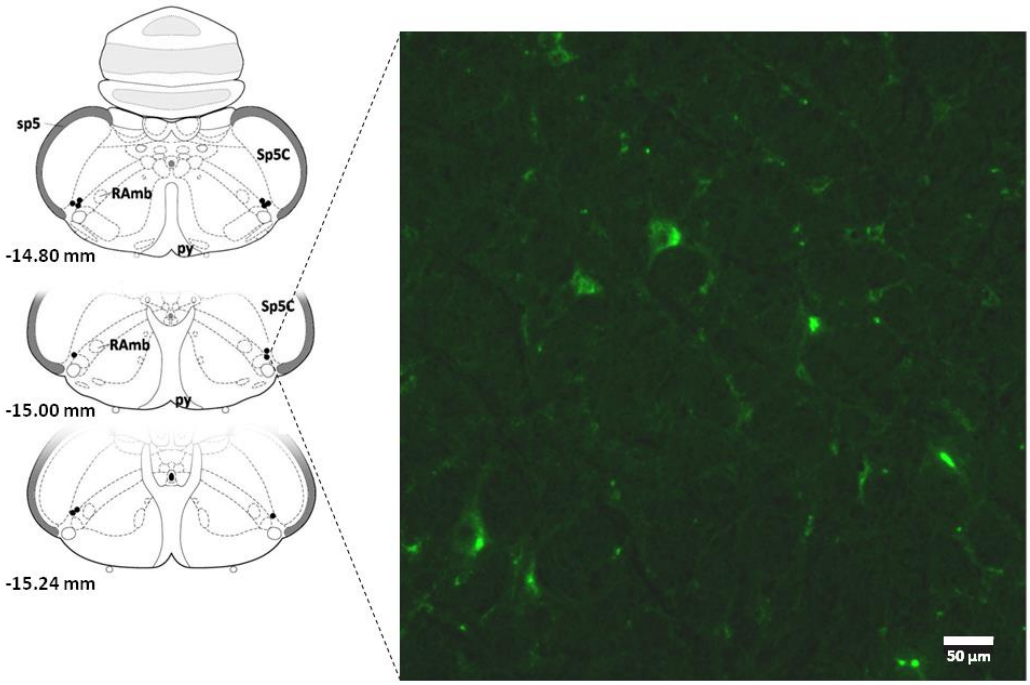


Fig. 3-10 – Localization of the MCPA microinjection sites (black circles) and lentiviral vector-mediated transduction of green fluorescent protein (GFP) in the MCPA; confocal microscope images of GFP-expressing cells in the MCPA *(bar: 50 µm*) following injection of lentiviral vector into this site. RAmb, nucleus retroambiguous; Py, pyramidal tract; Sp5, spinal trigemina nucleus.

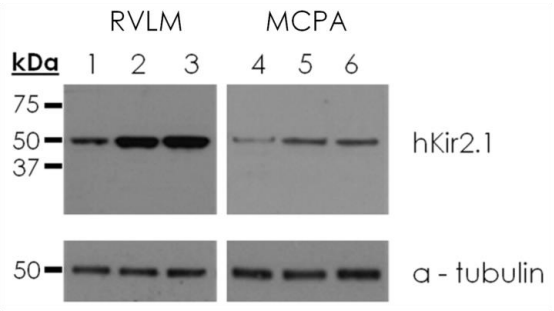


Fig. 3-11 - Western blot analysis of sham SHR (1, 4) and LVV-hKir2.1 microinjected SHR (2, 3, 5, 6) in RVLM and in MCPA. Results show an over expression of hKir2.1 in LVV-hKir2.1 microinjected SHR. α-tubulin was used as house keeping gene.

4. DISCUSSION

In the present work, our purpose was to depress chronically the activity of RVLM and MCPA neurones by the over-expression of K^+ channels to evaluate its consequences upon *long term* blood pressure regulation in conscious unrestrained SHR. For that, we over-expressed a human inward rectifying potassium channel (hKir2.1) under the control of a synapsin promoter that was neuron specific (Duale *et al.*, 2005a; Duale *et al.*, 2005b). Lentivirus was used as it induces sustained protein expression within neurones for months (Coleman *et al.*, 2003). In previous studies, Duale et al (2007) and Howorth et al (2009) showed that hKir2.1 over-expression hyperpolarized the membrane potential of cultured cathecolaminergic PC12 cells by ~10mV, which is expected to "electrically silence" neurones (Duale *et al.*, 2007; Howorth *et al.*, 2009). Similar over-expression strategies have been used to electrically silence neurones affecting development *in ovo* (Yoon *et al.*, 2008), neuronal discharge *in vivo* (Okada & Matsuda, 2008) and the ability of neurones to make and maintain connections *in vivo* (Yu *et al.*, 2004; Mizuno *et al.*, 2007; Hendy, 2010). This viral mediated approach has the advantage of being site specific and enabling over-expression in adulthood, avoiding the development of putative compensatory mechanisms associated with transgenic animals (Hendy, 2010).

Our results show that LVV-hKir2.1 microinjection in RVLM of conscious SHR lowered the frequency power of systolic blood pressure indicative of a reduction in sympathetic activity, however our interpretations of changes in SNA are indirect and based on spectral

analysis. This occurred coincident with a decrease in systolic (-39 mmHg), diastolic (-40 mmHg) and mean BP (-40 mmHg) at 60 days post-microinjection. Sham rats did not show decreases in BP during the recorded period. In contrast to the RVLM, LVV-hKir2.1 injection in the MCPA was without effect on arterial pressure over the same time frame. This might be explained by the fact that the MCPA is not dependent on the integrity of the RVLM as described above. This explanation also assumes that the MCPA does not contribute tonic activity to sympathetic motor outflow at rest in conscious rats. Thus, the physiological role that this cell group plays in circulatory control of normotensive and hypertensive animals remains to be fully determined.

Following all LVV microinjections into the RVLM and MCPA heart rate decreased. Since this was observed in all rat groups this change appears to be a function of time.

Despite the fall in arterial pressure in the SHR, LVV-hKir2.1 microinjection had no effect on the peripheral chemoreflex evoked cardiovascular and respiratory responses. This result was unexpected given the importance of the RVLM in mediating the peripheral chemoreceptor reflex evoked sympathoexcitation in acute anaesthetized rats (Koshiya & Guyenet, 1996a). We can hypothesize that the chronic depression of RVLM excitability could lead to neuronal plasticity and enhanced functional expression of peripheral chemoreflex pathways that bypass RVLM, such as those routing via the PVN, the lateral hypothalamus or the pre-limbic cortex (Owens & Verberne, 1996; Olivan *et al.*, 2001; Gabbott *et al.*, 2005).

The microinjection of the lentiviral vector (LVV-hKir2.1) in the RVLM did not evoke any change in the baroreflex sensitivity (BRS) in the SHR. This is already impaired in the SHR compared to normotensive rats, indicating a deficit in the vagal capacity to reduce heart rate (Verberne *et al.*, 1988; Widdop *et al.*, 1990; Minami & Head, 1993). However, impairment of BRS controlling heart rate was not associated with impairment of BRS controlling efferent sympathetic nerve activity in human hypertension (Grassi *et al.*, 1998) suggesting distinct reflex pathways. So, the impairment of BRS in the SHR is related to the efferent parasympathetic, vagal pathway. Due to these facts, we were not surprised that there are no changes in baroreflex gain, since this reflects cardiac reflex gain that is mainly determined by the activity of cardiac vagal motoneurons and these were not targeted in the present study.

Given that the peripheral chemoreflex and the baroreflex were tested under anesthesia the depressant effect of the agent cannot be neglected as it may exacerbate the reduced excitability of RVLM neurons and might alter the normal pattern of cardiovascular control (Korner, 1971). In particular, by stimulating the GABA-ergic system barbiturates will enhances the inhibitory pathway between the caudal ventrolateral medulla and the RVLM, further decreasing RVLM excitability. Future studies should focus on the baroreflex sympathetic vasomotor gain in SHR before and after LVV-hKir2.1 in the RVLM.

In summary, our data show that chronic expression of Kir2.1 in the RVLM of conscious unrestrained SHR caused a marked and sustained decrease in blood pressure without changes in the baro- and peripheral chemoreceptor reflex evoked responses in cardiovascular and respiratory parameters. This decrease was mostly due to a reduction in sympathetic output as revealed indirectly by a decrease in the power density of the LF band of SBP and by the decrease in LF/HF SBP balance. Our data is amongst the first to demonstrate the role of the RVLM in maintaining levels of arterial pressure in hypertension in conscious SHR. We suggest that a decrease in RVLM neuronal activity is an effective anti-hypertensive treatment strategy. Thus, the RVLM remains an area for novel therapeutic intervention for controlling BP long-term.

New findings and its importance under working hypothesis 2

Our data are amongst the first to demonstrate the role of the RVLM in maintaining levels of arterial pressure in hypertension in conscious SHR. Our results show that LVV-hKir2.1 expression of RVLM neurons caused a substantial and sustained decrease of blood pressure (SBP ~ 25 mmHg) reflecting a reduction in sympathetic output, as evidenced by the indirect decrease of the LF band. In contrast to the RVLM, LVV-hKir2.1 injection in the MCPA was without effect on arterial pressure and sympathetic output over the same time frame. These data strongly show that not all central sympathetic areas are involved in the sympathoexcitation observed under pathological conditions and that RVLM is a key area for the relay of sympathetic information. In accordance, the decrease of RVLM neuronal activity could be an effective anti-hypertensive treatment strategy, remaining RVLM as an area for novel therapeutic intervention to long-term control of BP.

Under review in:

Autonomic Neuroscience: basic & clinical

Essential role of RVL medullary neuronal activity in the long term maintenance of hypertension in conscious SHR by V Geraldes, N Gonçalves-Rosa, B Liu, JF Paton, and I Rocha

HYPOTHESIS 3

Hypertensive disease runs with modifications on peripheral organs, the most important being the highly vascular ones like the brain, kidney and heart and vessels. Despite the earliest changes in these hypertensive target organs are largely compensatory in nature with time, in particular if the patient is not treated, they lead to functional compromises like left ventricular hypertension, stroke or renal failure. Recent studies have shown that pharmacological therapeutics with ACE inhibitors or beta blockers is able not only to delay the progression of target organs damage but to evoke reverse remodelling. In accordance, the following working hypothesis was built:

Will the chronic depression of brain sympatho-excitatory regions activity induce major signalling changes in hypertensive target organs condition?

1. INTRODUCTION

Arterial Hypertension (AHT) and its development are associated with structural, functional, genomic and transcriptomic alterations in several organs, in particular in the hypertensive target organs like brain, heart, kidney and vasculature, all of them contributing to cardiovascular risk. In conditions when AHT is uncontrolled, genomic expression and transcriptomic alterations can evoke changes in different signal-transducing cascades, thus, accelerating organ damage which ultimately results in organ failure and secondary disease such as stroke, cardiac ischemia and nephropathy. It is well recognised that elevated blood pressure can also cause left ventricular hypertrophy, aortic stiffness, atherosclerotic plaques and microvascular disease that may render AHT more difficult to control (Muiesan ML, 2013; Raizada, 1993; Schork, 1995).

Significant progress in molecular biology has demonstrated that AHT develops as a complex pathological state with a genetic background involving various hormonal and neuronal systems.

In normal cardiovascular function, angiotensin II and endothelin are implicated in the regulation of normal cardiovascular function, including regulation of peripheral artery resistance, vasodilation, vasoconstriction and vascular tone. These functions are exerted by signaling pathways through G protein-coupled receptors being well established that changes in these signaling pathways are contributory factors for hypertension.

In particular, to angiotensin II and the renin-angiotensin system, it is well established that this hormonal system is elevated in several experimental models of AHT, as well as human essential AHT (Cowley 1992; Reinhart et al. 1995; Lenkei et al. 1997; Weir and Dzau 1999; de Gasparo et al. 2000; Lifton et al. 2001; Doris 2002). An elevated RAAS impacts blood pressure directly via vasoconstriction and sodium retention, through generation of angiotensin II (Ang II), as well as indirectly through increased reactive oxygen species (ROS), altering redox signaling and increased sympathetic outflow (Collett J, 2013).

More recently the existence of the local or tissue RAAS has been established and is thought to participate in cardiovascular regulation (Shan et al., 2004; Klett C, 1993; Nyui N, 1997, Tamura K, 1995; Griendling KK, 1993; Dzau VJ, 1994). This local RAAS may play an important role in hypertension and may exist and function in the heart, brain, adrenal gland, kidney, blood vessel wall, and adipose tissue (Nyui N, 1997; Tamura K, 1996).

However, the exact role of this local system is not clear, since there are doubts about the physiologically relevance of some components of the RAAS, but it is interesting to speculate that a local RAAS may increase the effects of Ang II on a specific tissue in a particular physiological and pathophysiological processes, such AHT (Tamura K, 1996). Angiotensin-converting enzyme (ACE) inhibitors can lower the blood pressure in spontaneously hypertensive rats (SHR) mainly by reducing production of Ang II and decreasing bradykinin degradation (Gohlke P, 1994; Johnson CI, 1994). In fact, a 4-week period of ACE inhibitor treatment in young SHR is sufficient to prevent the full expression of genetic hypertension and cardiovascular hypertrophy and that Ang II might be important in the development of hypertension in this animal model of AHT (Harrap SB, 1990).

Endothelial cells are also involved in blood pressure control by releasing vasoactive and trophic factors that regulate vascular tone being nitric oxide (NO) and endothelin 1 (ET-1), two endothelial factors that are particularly involved in this regulation. Endothelial NO produced through the interference of eNOS is the main vasodilator factor that causes relaxation of the vascular smooth muscle. In hypertension, endothelial dysfunction has been related to an increased NO breakdown by reactive oxygen species (ROS) and a reduced NO production by eNOS resulting in a reduced vasodilator capacity of vessels (Marín-García et al., 2011). Together with these changes on NO availability, the dysfunctional endothelial cells also produce a series of vasoconstrictor factors which include endothelin 1. At vascular level, ET-1 binds to two types of endothelin receptors, type A and B. The type A receptors are mainly located in the smooth muscle cells and stimulate vascular contraction; on the other hand, ETB-receptors are abundant on endothelial cells and mediate NO release facilitating vasodilation. In hypertension, the ETB-receptors fail to increase NO-mediated vasodilation being the overall effect effect, an increase of vessels constriction due to the stimulation of ET-A receptors by ET-1 (Penna et al., 2006; Kohan et al., 2011; Ohkita et al., 2012; Kaoukis et al., 2013; Moorhouse et al., 2013).

Studies from our laboratory have shown that reducing the neuronal activity in either the paraventricular nucleus of the hypothalamus (PVN) or rostral ventrolateral medulla (RVLM) via chronic over expression of an inwardly rectifying potassium channel (hKir2.1) of SHR resulted in a long term and persistent decrease of blood pressure and sympathetic activity (Geraldes et al., 2014a; Geraldes et al, 2014b).

Since the aetiology of the signalling changes in hypertensive target organs is not yet fully understood, in the present work, we studied tissue-specific mRNA expression genes of hypertensive target organs following the persistent decrease of blood pressure and sympathetic output to clarify the causal relationship between the decrease of blood pressure values and the reverse signalling phenomena in hypertensive target organs tissue.

2. MATERIALS AND METHODS

All the experimental procedures were in accordance with the European and Portuguese Law on animal welfare and had the approval of the ethic committee of the Faculty of Medicine, University of Lisbon, Portugal. WKY rats (n=7) and SHRs (n=14), males, aged 12 weeks and weighing 363±8g, were used. Animals, synchronized for a 12:12h light-dark cycle (light on at 7am, light off at 7pm), were housed individually and allowed to freely move in standard plastic cages. Food and water were available *ad libitum*.

2.1. Lentiviral treatment

SHRs were divided into 2 groups according to the content of the microinjection: LVV-hKir2.1 (n=7) and LVV-eGFP (n=7). Rats were implanted with radio-telemetry probes (DSI) in the abdominal aorta under anaesthesia (sodium pentobarbitone, 60mg/Kg, IP) and were allowed to recover for 15 days.

Two weeks after the probes were implanted, SHR rats were placed in a stereotactic frame (Kopf Instruments) and a craniotomy performed using our previously determined coordinates for LVV-hKir2.1 (LV-TREtight-Kir-cIRES-GFP 5.4x10E9 and LV-Syn-Eff-G4BS-Syn-Tetoff 6.2x10E9 in a ratio 1:4) bilateral microinjections (0.05µl) into the PVN (B: -1.6mm, L: -/+1.41mm, D: 7.4mm; pipette angle: 10°) or RVLM (B: -12.5mm, L: 2.1mm, D: 8mm) (G & C, 1986). Sham rats were microinjected in the same region with LVV-eGFP (LVV-TREtight-GFP 5.7x10E9 and LVV-Syn-Eff-G4BS-Syn-Tetoff 6.2x10E9 in a ratio 1:4). Animals were allowed to recover and monitored by telemetry for 60 days. Heart rate (HR) and blood pressure (BP; systolic, diastolic and mean) were recorded continuously. The microinjection was not applied to WKY rats.

2.2. Organ tissue processing, RNA isolation and cDNA Synthesis

At the end of the experimental protocol described above, animals were killed with an overdosis of anesthesia (pentobarbital, 50 mg/kg, i.v.). The target organs (heart, vessel and kidney) were excised, immediately frozen separately in liquid nitrogen and stored at −80 ◦C for subsequent RNA isolation.

The heart, carotid artery and kidney samples of individual LV-treated SHR, Sham SHR and WKY rats were grinded with a mortar and pestle in liquid nitrogen and approximately 50mg of powdered tissue was homogenized in Tri Reagent® Solution (Ambion) for total RNA isolation according to the manufacturer's instructions. RNA concentration was estimated by measuring the absorbance at 260nm and its purity assessed by determining the 260/280nm absorbance ratio using NanoDrop 1000A (Thermo Scientific).

First strand cDNA was synthesized from 1µg of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions.

2.3. Quantitative real-time PCR analysis

Oligonucleotide primers were designed with Primer Express ® Software Version 3.0 (Applied Biosystems) according to the recommended parameters for quantitative assays, based on the mRNA sequences obtained from the Rat Genome Database. Gene ID and oligonucleotide sequences are listed in Table 3.5. The chosen genes are known to be involved in various processes including blood pressure regulation, renin-angiotensin system, nitric oxide metabolism and signalling, vasoconstriction/vasodilatation, osmotic shock, ion transport, nitric oxide metabolism, hypoxia response and vasotone.

Real-time PCR reactions were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using Fast SYBR® Green Master Mix reagents, following the manufacturer's protocol. Immediately after amplification, melt curve analysis was performed in order to check PCR reactions for primer-dimer artifacts and to ensure specificity.

Table 3.5 - Primers and respective sequences designed for Real Time PCR

Gene name (Symbol) **Accession number**	**Primer sequence** **Forward/Reverse**
Angiotensinogen (Agt) NM_134432	CCCTGAGCAGTCCGTTCCT AAAGTGCAGCGCACCTGAGT
Angiotensin II receptor, type 1a (AT1a) NM_030985	GCCAGGGCAGCCTCTGA TCCTGAGGCAGGGTGAATG
Angiotensin II receptor, type 1b (AT1b)	CCTCCGCCGCACGAT

NM_ 112271	CCATTAGCCAGATGATGATGCA
Angiotensin II receptor, type 2 (AT2) NM_012494	TGCTGTTGTGTTGGCATTCA ATCCAAGAAGGTCAGAACATGGA
ATPase, Ca++ transporting, type 2C, member 1 (Atp2c1) NM_131907	TGGAACCCTGACGAAGAATGA GCATGCAGGCCGTCTGA
Endothelin 1 (ET-1) NM_012548	TGGAGGCCATCAGCAACAG AGTTCCGCTTTCAACTTTGCA
Nitric oxide synthase 3, endothelial cell (Nos3) NM_021838	TCTTTCGGAAGGCGTTTGAC CTCTAGGGATACCACATCGTATTCATC
Renin (Ren) NM_012642	CTGCTCAGGCTGTTGATGGA CACCCTCTGGGAGAGAATGTG
Troponin T type 2 (cardiac) (Tnnt2) NM_012676	CAGGAAGCGCATGGAGAAG TCGAAGTGAGCCTCGATCAGA
Tropomyosin 1, alpha (Tpm1) NM_019131	GGCCAAGCACATTGCTGAA GCTTACGGGCCACCTCTTC
Tropomyosin 2, beta (Tpm2) NM_001024345	TAACCTGTCCCGGGTGCAT GCGAGCGGTGAAGAGTAGGTA
myosin, heavy chain 6, cardiac muscle, alpha (Myh6) NM_017239	ACAGAGTGCTTCGTGCCTGAT CAGTCACCGTCTTGCCGTTT
myosin, heavy chain 7, cardiac muscle, beta (Myh7) NM_017240	CAGCCTACCTCATGGGACTGA TGACATACTCGTTGCCCACTTT
Actin, beta ActB/ NM_031144	ACCAGTTCGCCATGGATGAC TGCCGGAGCCGTTGTC

The relative quantification of gene expression in treated SHR, sham SHR and WKY samples was determined by comparative C(t) method, using the normotensive WKY group as a calibrator to estimate the relative amount of mRNA in both SHR groups. The mRNA level of all samples was normalized against an endogenous control (β-actin). The fold-change for the SHR samples relative to WKY was calculated by $2^{-\Delta\Delta C(t)}$, where $\Delta\Delta C(t) = \Delta C(t)SHR - \Delta C(t)WKY$,and $\Delta C(t)C(t)targetgene - C(t)endogenous\ control$.

2.4. Physiological data acquisition and analysis

Telemetric data were acquired at 1KHz (Powerlab, ADInstruments). Mean values of BP (systolic, diastolic and mean) were directly extracted. From the interpolation of pulse pressure peaks were derived HR and Respiratory Rate (RespR), the last one after the application of Fourier analysis to the interpolated signal.

Systolic BP and RR interval data were analyzed (period of 3 minutes) in the frequency domain (Fast Fourier Transform, FFT), using the in-house software Fisiosinal (Tavares, 2011b), to evaluate sympathetic (Low Frequency band, LF, 0.15-0.6Hz of SBP) and parasympathetic (High Frequency band, HF, 0.6-2.0Hz of HR) activity over time (M Malik, 1996; Marques-Neves *et al.*, 2004).

2.5. Statistical analysis

Comparisons between groups for the same period and also comparisons within the same group, before and after the microinjections were performed. For the statistical analysis, Student's t test for paired data and ANOVA for comparisons between inter-groups were used. All data were expressed as mean±SEM and passed the normality test. Significance was taken as $P < 0.05$.

3. RESULTS

3.1 Effect on blood pressure, heart rate and sympathetic output of potassium channels overexpression in the PVN and RVLM

At 60 days post-injection, LVV-hKir2.1 expression in PVN produced a time-dependent and significant decreases in systolic (158±3 to 132±6 mmHg $p<0.05$) and diastolic (135±4 to 113±5 mmHg $p<0.05$) and mean BP (142±3 to 120±5mmHg, $p<0.05$). These BP changes were accompanied by a lowering of HR (295±3 bpm, $p=0.099$).

LVV-hKir2.1 expression in RVLM produced a time dependent decrease in of systolic (155±3 to 116±8 mmHg; SBP), diastolic (130±3 to 90±12 mmHg), mean (138±3 to 98±10 mmHg) BP and HR (310±4 to 293±6) 60 days post-injection ($p<0.01$).

At the same time, PVN SHR LVV-eGFP group were showing increased values of systolic (174±10 mmHg, $p>0.05$), diastolic (149±11 mmHg, $p>0.05$) and mean BP (157±10 mmHg,

p>0.05) together with a significantly HR decreased (285±6 bpm, p<0.01). The RVLM SHR LVV-eGFP group showed increased values of systolic (168±9 mmHg, p>0.05), diastolic (148±9 mmHg, p>0.05) and mean BP (155±9 mmHg, p>0.05) together with a decrease in HR (292±4 bpm, p>0.05). This profile of BP and HR changes in SHR sham was expected and consistent with their developmental trend (Dickhout & Lee, 1998) .

SHRs showed putative evidence for an overall decrease of cardiovascular autonomic outflow at 60 days after the treatment. Indeed, a strong decrease in sympathetic output expressed by LF_{sBP} band power (from 0.79±0.13 to 0.42±0.09 $mmHg^2$, p<0.05), was observed suggesting a reduced sympathetic vasomotor tone. Low frequency spectra of SBP in RVLM LVV-hKir2.1 SHR decreased from 0.72±0.09 to 0.42±0.10 $mmHg^2$.

In contrast, at 60 days the LF_{sBP} for PVN SHR LVV-eGFP was 0.86±0.21 (p>0.05) and for RVLM SHR LVV-eGFP was 0.86±0.16 (p>0.05).

No significant changes in BP, HR, RespR and autonomic outflow were observed in WKY rats during the 60 days duration of the experimental protocol.

3.2. Gene expression changes in heart, vessels and kidney and tissue induced by LVV-hKir2.1 treatment

The present study was designed to extensively identify 'signature' genes that could be altered by LVV-hKir2.1 treatment in individual end-organs.

Using RT-PCR, the expression profile of 17 genes was analyzed in the heart, vessels and kidneys in treated SHR, Sham SHR and sham WKY rats. The genes quantified for each target organ sample are shown in table 3.6. The results are shown as below separately for each gene, tissue and microinjected area (Figure 3-12, to 3-17; Table 3.7, 3.8 and 3.9).

Table 3.6 - Selected genes and samples analyzed

Gene	Target Organ Sample
angiotensinogen (Agt)	Kidney
angiotensin II receptor, type 1a (AT1a)	Kidney

angiotensin II receptor, type 1b (AT1b)	Kidney, Heart
angiotensin II receptor, type 2 (AT2)	Kidney
ATPase, Ca++ transporting, type 2C, member 1 (Atp2c1)	Kidney, Heart
endothelin converting enzyme 1 (Ece1)	Carotid
endothelin 1 (ET-1)	Kidney, Heart, Carotid
endothelin 2 (ET-2)	Carotid
endothelin receptor type A (Ednra)	Carotid
endothelin receptor type B (Ednrb)	Carotid
nitric oxide synthase 3, endothelial cell (Nos3)	Kidney, Heart
renin (Ren)	Kidney
troponin T type 2 (cardiac) (Tnnt2)	Heart
tropomyosin 1, alpha (Tpm1)	Heart
tropomyosin 2, beta (Tpm2)	Heart
myosin, heavy chain 6, cardiac muscle, alpha (Myh6)	Heart
myosin, heavy chain 7, cardiac muscle, beta (Myh7)	Heart
actin, beta (Actb)	Kidney, Heart, Carotid

3.2.1. Expression changes in the heart

Comparing the mRNA expression in treated SHR with the WKY rats: among the 9 genes studied only 1 was down-regulated – myosin 7 (3,6 fold) in RVLM treated SHR (Figure 3-12, table 3.7).

Comparing the mRNA expression in treated SHR with the SHR sham: AT1 (3,9 fold and 3,8 fold), ATP2C1 (2,6 fold and 2,5 fold) and Tnnt2 (2,7 fold and 2,0 fold) were down-

regulated in treated PVN SHR and in treated RVLM SHR, respectively. Also, Tpm2 was down-regulated (3,0 fold) in treated RVLM SHRs (Figure 3-13, table 3.7).

Table 3.7 - mRNAs Expression in the heart of SHR after the treatment with LVV-hKir2.1 in the PVN and in the RVLM relative to WKY group or to SHR SHAM group. *p<0.05; **p<0.01.

PVN SHR Genes	Fold change relative to WKY	Fold change relative to SHAM	RVLM SHR Genes	Fold change relative to WKY	Fold change relative to SHAM
AT1	1,36	0,26*	**AT1**	1,38	0,26*
Atp2C1	0,74	0,38**	**Atp2C1**	0,78	0,40**
ET-1	1,25	2,05	**ET-1**	1,65	2,35
Myh6	0,62	0,85	**Myh6**	0,49	0,68
Myh7	0,43	0,76	**Myh7**	0,28*	0,72
NOS3	0,57	1,19	**NOS3**	0,98	2,05
Tnnt2	0,70	0,36*	**Tnnt2**	0,76	0,50*
Tpm1	0,78	0,43	**Tpm1**	0,77	0,42
Tpm2	1,34	0,99	**Tpm2**	1,08	0,34*

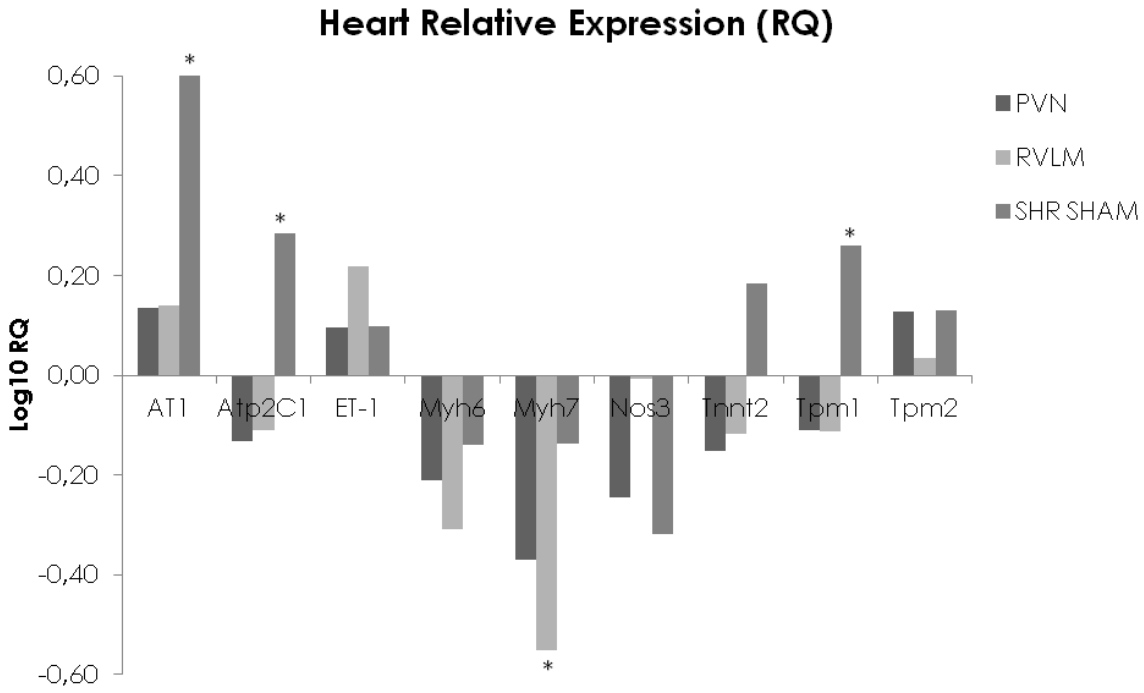


Figure 3-12 – mRNA expression in the heart of treated PVN and RVLM SHR and SHR SHAM relative to WKY rats. AT1, angiotensin II receptor type 1; Atp2C1, ATPase, Ca++ transporting, type 2C, member 1; ET-1, endothelin 1; Myh6, Myosin 6; Myh7, Myosin 7; Nos3, Nitric oxide synthase 3, endothelial cell; Tnnt2, Troponin T type 2; Tpm1, Tropomyosin 1, alpha; Tpm2, Tropomyosin 2, beta. *p<0.05.

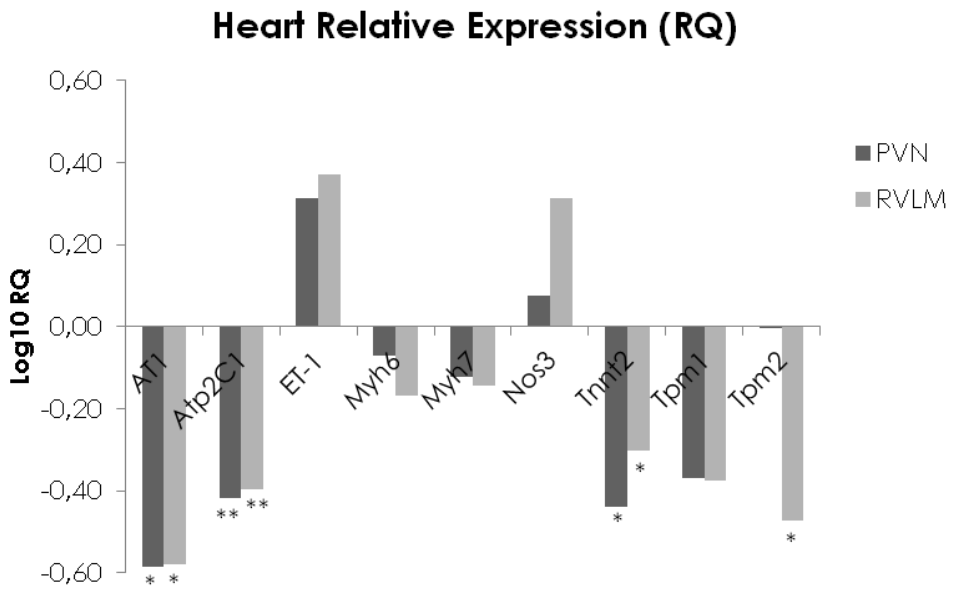


Figure 3-13 – mRNA expression in the heart of treated PVN and RVLM SHR relative to SHR SHAM group. AT1, angiotensin II receptor type 1; Atp2C1, ATPase, Ca++ transporting, type 2C, member 1; ET-1, endothelin 1; Myh6, Myosin 6; Myh7, Myosin 7; Nos3, Nitric oxide synthase 3, endothelial cell; Tnnt2, Troponin T type 2; Tpm1, Tropomyosin 1, alpha; Tpm2, Tropomyosin 2, beta. *p<0.05; **p<0.01.

3.2.2. Expression changes in the kidney

Comparing the mRNA expression in treated SHR with the WKY rats: in the PVN and in the RVLM, between the 8 genes studied only 2 were up-regulated – endothelin 1 (3,0 fold and 5,3 fold) and AT2 (2,2 fold and 8,3 fold), respectively (Figure 3-14, table 3.8).

Comparing the mRNA expression in treated SHR with sham SHR rats: 3 genes were up-regulated – Angiotensinogen (1,9 fold and 2,7 fold), AT2 (11,1 fold and 42,1 fold) and ET-1 (8,5 fold and 14,7 fold) in treated PVN SHR and in treated RVLM SHR, respectively (Figure 3-15, table 3.8).

Table 3.8 - mRNAs Expression in the kidney of SHR after the treatment with LVV-hKir2.1 in the PVN and in the RVLM, relative to WKY group or to SHR SHAM group. *p<0.05; **p<0.01; ***p<0.001

PVN SHR Genes	Fold change relative to WKY	Fold change relative to SHAM	RVLM SHR Genes	Fold change relative to WKY	Fold change relative to SHAM
Agt	1,09	1,91**	**Agt**	1,51	2,67**
AT1a	1,10	0,80	**AT1a**	1,16	0,85
AT1b	0,85	0,59	**AT1b**	1,60	1,10
AT2	2,20*	11,10*	**AT2**	8,34*	42,10***
Atp2C1	1,20	1,55	**Atp2C1**	1,01	1,31
ET-1	3,06***	8,47***	**ET-1**	5,30***	14,69***
NOS3	0,62	0,50	**NOS3**	0,83	0,66
Ren	1,21	0,71	**Ren**	1,52	0,89

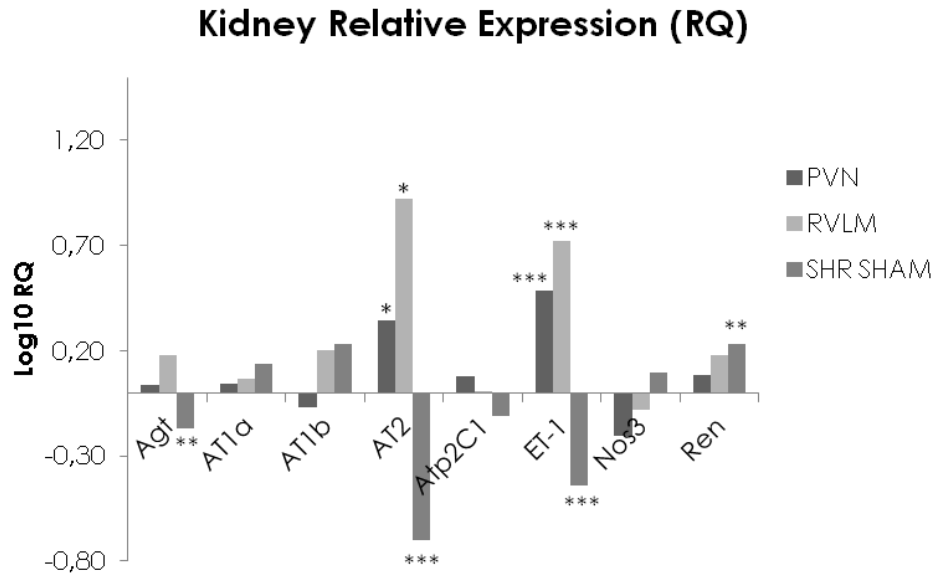


Figure 3-14 – mRNA expression in the kidney of treated PVN and RVLM SHR and SHR SHAM relative to WKY rats. Agt, Angiotensinogen; AT1a, angiotensin II receptor type 1a; AT1b, angiotensin II receptor type 1b; AT2, angiotensin II receptor type 2; Atp2C1, ATPase, Ca++ transporting, type 2C, member 1; ET-1, endothelin 1; Nos3, Nitric oxide synthase 3, endothelial cell; Ren, Renin. *p<0.05; **p<0.01; ***p<0.001.

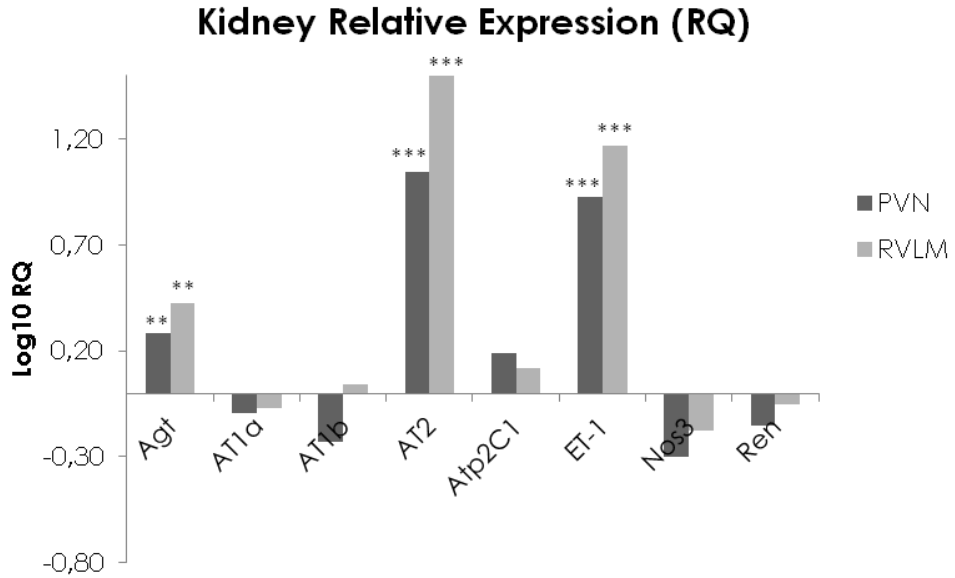


Figure 3-15 – mRNA expression in the kidney of treated PVN and RVLM SHR relative to SHR SHAM group. Agt, Angiotensinogen; AT1a, angiotensin II receptor type 1a; AT1b, angiotensin II receptor type 1b; AT2, angiotensin II receptor type 2; Atp2C1, ATPase, Ca++ transporting, type 2C, member 1; ET-1, endothelin 1; Nos3, Nitric oxide synthase 3, endothelial cell; Ren, Renin. *p<0.05; **p<0.01; ***p<0.001.

3.2.3. Expression changes in the carotid artery

In treated PVN SHR, among the 5 genes studied only 1 was up-regulated (1,7 fold) - endothelin receptor type A (Ednra) –compared with WKY rats (Figure 3-16, table 3.9). Comparing the mRNA expression in treated PVN SHR with sham SHR rats, 1 gene was a down-regulated - endothelin converting enzyme 1 (3,0 fold) - and 1 gene was up-regulated - endothelin-2 (ET-2) (4,8 fold) (Figure 3-17, table 3.9).

In treated RVLM SHR, 2 genes were up-regulated – endothelin converting enzyme 1 (Ece1; 2,1 fold) and endothelin receptor type A (Ednra; 1,5 fold) - compared with WKY rats (Figure 3-16, table 3.9). Comparing with sham SHR group there was an up-regulation of Ece1 (2,0 fold) and ET-2 (3,0 fold) in RVLM treated SHR (Figure 3-17, table 4).

Table 3.9 - mRNAs Expression in the carotid artery of SHR after the treatment with LVV-hKir2.1 in the PVN and in the RVLM. relative to WKY group or to SHR SHAM group. *p<0.05

PVN SHR Genes	Fold change relative to WKY	Fold change relative to SHAM	RVLM SHR Genes	Fold change relative to WKY	Fold change relative to SHAM
Ece1	0,35	0,33	**Ece1**	2,10*	1,98*
ET-1	1,54	1,09	**ET-1**	1,38	0,97
ET-2	3,29	4,76*	**ET-2**	2,06	2,99*
Ednra	1,70*	2,55	**Ednra**	1,49*	1,75
Ednrb	2,26	2,94	**Ednrb**	1,75	2,27

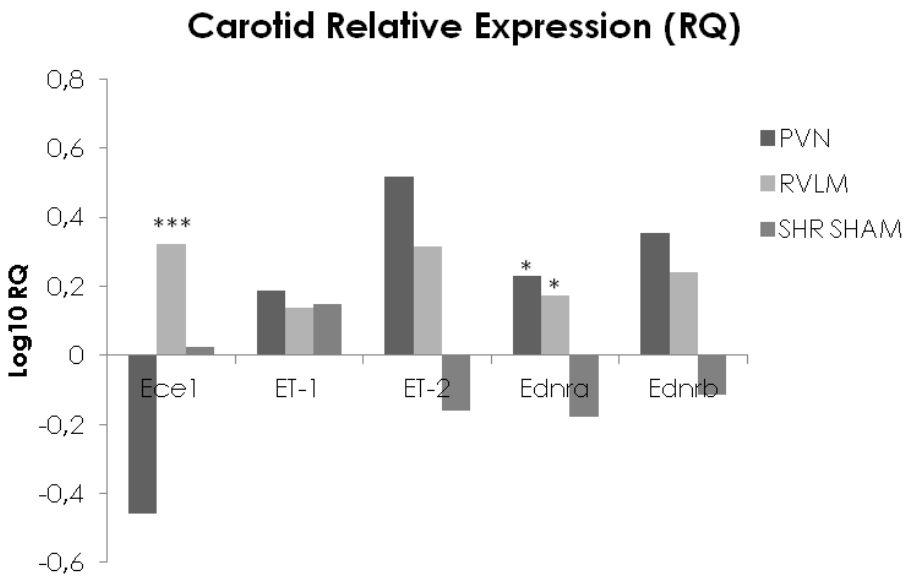


Figure 3-16 – mRNA expression in the carotid artery of treated PVN and RVLM SHR and SHR SHAM relative to WKY rats. Ece1, endothelin converting enzyme 1; ET-1, endothelin 1; ET-2, endothelin 2; Ednra, endothelin receptor type A; Ednrb, endothelin receptor type B. *p<0.05;***p<0.001.

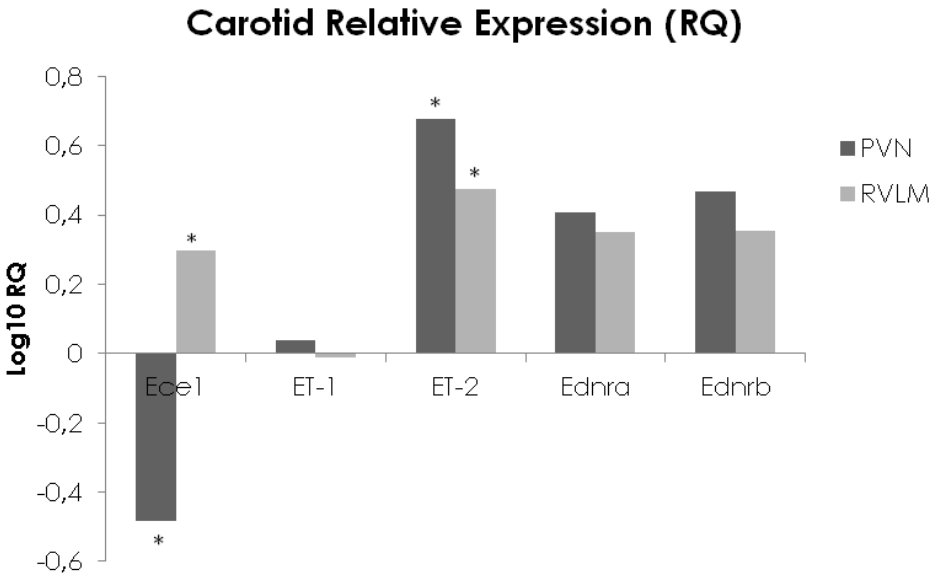


Figure 3-17 – mRNA expression in the carotid artery of treated PVN and RVLM SHR relative to SHR SHAM group. Ece1, endothelin converting enzyme 1; ET-1, endothelin 1; ET-2, endothelin 2; Ednra, endothelin receptor type A; Ednrb, endothelin receptor type B. *p<0.05.

4. DISCUSSION

We previously showed that a decrease in PVN and RVLM neuronal excitability of SHR caused a sustained decrease in blood pressure and sympathetic output (Geraldes *et al.*, 2013). In order to determine if the decrease in cell excitability induced by the chronic overexpression of hKir2.1 channels in the PVN and RVLM induced reverse remodeling in target organs, we evaluated gene expression changes in the heart, vessel and kidneys, the major end-organs associated with arterial hypertension (HTA).

Our main finding of the present study was that treatment of LVV-hKir2.1 promotes the remodeling process in target organs, as showed by the different regulation of gene expressions in cardiac and renal tissues of treated SHR.

a) Heart and vessel

Cardiac hypertrophy is one of the major hypertension-induced pathological consequences. Taking in to account the 9 genes evaluated in the present study, only 1 was different from WKY rats – the myosin heavy chain β (β-MHC). The β-MHC 7 (V3 cardiac myosin) was downregulated (3,6 fold) in treated RVLM SHR and are involved in regulation of contractility or hypertrophy. In normal cardiac tissue the myosin heavy chain α (α-MHC; v1 cardiac myosin) is expressed predominantly, but in models of cardiac hypertrophy, the β-MHC has a higher expression than the α-MHC (Mercadier *et al.*, 1981; Compagno *et al.*, 2001) showed that the β-MHC was markedly expressed in the ventricle of 15-week old SHR vs WKY. Hence, our results showed that the LVV-treatment decreased the β-MHC expression in RVLM SHR in comparison to the WKY rats, similar to Ang II receptor type 1 antagonist that promotes the decrease in BP and downregulate MHC in the aorta of SHR (Fujii *et al.*, 1999).

There was no significant difference between treated SHR and WKY rats in the expression of AT1 receptor, ATPase Ca^{2+}, Endothelin-1, Myosin 6, Nitric oxide synthase 3, Troponin T2, Tropomyosin 1 (α) and Tropomyosin 2 (β) in the heart. There are several studies showing differences in gene expression in SHR vs WKY rats, which mean that there is a remodeling process in the heart of treated SHR. In fact, AT1 receptor is up-regulated and there is evidence that indicate that the decrease in Ca2+ ATPase expression occur in prominent hypertrophy and in the failured heart, however it was shown that the

expression of SR Ca2+-ATPase was not down-regulated in the heart of 11 week old SHR (Ohta *et al.*, 1995). In addition, Troponin is up-regulated and NO synthase is downregulated in SHR in comparison with the WKY rats (Bauersachs *et al.*, 1998; Piech *et al.*, 2003).

Both treated SHR had a down-regulation of AT1 (3,9 fold and 3,8 fold), ATP2C1 (2,6 fold and 2,5 fold) and Tnnt2 (2,7 fold and 2,0 fold) in the heart. The RVLM SHR group has also showed a down-regulation of Tpm2 when compared to the SHR sham group. Thus, it seems that there is a remodeling process in the hypertrophied heart of this animal model, since there is a continued improvement in gene expression in the heart of these animals, approaching the normality.

However in our study we did not include a measurement of wall stress, which is influenced by pressure, chamber radius and wall thickness.

In the carotid artery, there is an up-regulation of endothelin receptor type A (Ednra) in treated PVN and RVLM SHR in comparison to WKY rats (1,7 fold and 1,5 fold, respectively). We have also found an up-regulation (2,1 fold) of endothelin converting enzyme 1 (Ece1) - in treated RVLM SHR compared with WKY rats.

When compared to the sham group, the treated PVN and RVLM SHR showed an up-regulation (4,7 fold and 3,0 fold, respectively) of endothelin-2 (ET-2). In the PVN SHR group there is also a down-regulation of Ece1 (3 fold) and in the RVLM SHR group there is an up-regulation of Ece1 (2,0 fold). So it seems that the vasculature is trying to compensate the decrease in blood pressure values obtained by central manipulation though the up-regulation of Edrna (in the PVN and RVLM SHR group) and Ece1 (in the RVLM SHR group) .

b) Kidney

In the kidneys of PVN and RVLM treated SHR for the 8 genes studied only 2 were up-regulated -ET-1 and AT2 - with a fold change of 3,0 and 2,2 for PVN SHR and 5,3 fold and 8,3 fold for RVLM SHR, respectively, when compared to WKY rats. In the rest of the genes analyzed there were no differences between the two groups: Angiotensinogen, AT1a, AT1b, ATPase Ca^{2+}, Nitric oxide synthase 3 (Nos3) and Renin.

ET-1 is a potent vasoconstrictor but the role of ET-1 in SHR remains unclear. Hughes et al found that SHR and aged-matched WKY rats had no difference in renal ET-1 levels until AHT appeared. After the development of AHT the SHR had a significantly reduced ET-1 in the urine and in the outer and inner medulla of the kidney (Hughes *et al.*, 1992; Largo *et al.*, 1997). Hence, the up-regulation of ET-1 found in the kidneys of treated SHR relative to normotensive rats (WKY) and to Sham SHR seems not to participate in the progression of AHT in this animal model.

(Wu *et al.*, 1994), observed in the kidneys of SHR an up-regulation in Ang II receptor AT-1 in comparison to WKY rats. Therefore, we can assume that the LVV-treatment in SHR can reduce the mRNA levels of AT-1 in the kidneys, since these are similar to the mRNA AT-1 levels in WKY rats, thus close to normality, which is also a limitation to the vasoconstrictive effect of Ang II.

Ang-(1–7) has been shown to directly downregulate another RAS component, the AT-1 receptor, in cultured vascular smooth muscle cells (Clark *et al.*, 2001). Our results showed an up-regulation in AT-2 receptor in the treated SHR, thus contradicting the biological effects of AT1 receptor activation, promoting vasodilation, growth inhibition and cell differentiation (Suzanne Oparil, 2003). In the present study we didn´t analyze the Ang-(1-7) expression, so, we can not speculate about the vasodilatation action of Ang-(1-7).

Cosentino *et al.* showed that long-term treatment with the AT1R antagonist, losartan, in SHR promotes a significantly increase in AT2R mRNA in thoracic aortas, supporting our finding about the beneficial up-regulation of AT2R mRNA in the kidney of treated SHR (Cosentino *et al.*, 2005).

An increased expression of AT2 by LVV-treatment may be a possible way to normalize or improve the peripheral chemosensitivity and result in decrease of sympathetic activation and blood pressure in SHR (Geraldes *et al.*, 2013). In patients with systemic arterial hypertension, the statin therapy has the same effect though the down-regulation of AT-1 receptors. There are studies showing the increase in NO synthase in SHR in comparison to WKY rats (Vaziri *et al.*, 1998; Fernández *et al.*, 2003), so it seems that the LVV-treatment tends to decrease NO synthase expression in the kidney of treated SHR similar to WKY rats.

According to several studies, there is an increase in renin mRNA expression in the kidneys of SHR (Antonaccio *et al.*, 1984; Samani *et al.*, 1989; Nakamura & Johns, 1995). The renin-angiotensin system in the kidney plays an important role in the regulation of hemodynamic and tubular functions and it is established that renin, angiotensinogen, and angiotensin converting enzyme can produce angiotensin II locally (Johns, 1989). In the kidney, the renin release is regulated by the renal sympathetic nerves (mediated by β1-adrenoceptors); by the pressure-sensitive renal baroreceptor; and by the macula densa, which is responsive to tubular fluid composition (Skøtt & Jensen, 1993).

There is evidence of a relation between renal sympathetic nerve activity and the production and release of renin. In fact, renal denervation in the rat blunted the increase in renal renin mRNA after long-term ureteral obstruction (el-Dahr et al., 1991). Similarly, renal renin mRNA levels were lower in denervated than innervated kidneys (Page et al., 1992). These studies showed that tonic activity in the renal nerves could elevate renin gene expression. Therefore, we can speculate that the decrease in sympathetic nerve activity promoted through the LVV-treatment can decrease the renin expression in the kidneys of treated SHR, since mRNA levels of renin are similar to WKY rats.

There is a positive correlation between the angiotensinogen levels and the blood pressure found in rats and in humans (Dzau VJ, 1989, El-Dahr SS, 1991, Page WV, 1992, Nakamura A, 1994; Bruna RD, 1993). Other studies using the rat and human angiotensinogen genes have suggested that the transcriptional mechanism of the angiotensinogen gene is involved in the pathogenesis of AHT (Ingelfinger *et al.*, 1986; Ingelfinger *et al.*, 1990; Nakamura & Johns, 1995). In addition, the angiotensinogen-deficient mice doesn´t produce angiotensinogen and are hypotensive, what shows the impact of angiotensinogen in the maintenance of BP and in the development of AHT (Pratt *et al.*, 1989; Tanimoto *et al.*, 1994).

The kidneys of the SHR contain lower levels of angiotensinogen mRNA compared with the WKY rats (Pratt *et al.*, 1989). There is also evidence that low levels of renal sympathetic activity may increase angiotensinogen gene expression (Nakamura & Johns, 1994). In our study, the LVV-treatment decreased the sympathetic activity and increased the angiotensinogen mRNA in the kidneys of treated PVN and RVLM SHR compared to sham SHR (1,9 fold and 2,7 fold) and to similar levels found in WKY rats. One hypothesis is that

the decrease in sympathetic activity found after the LVV-treatment could be the cause for the increase in angiotensinogen gene expression found in the kidney of treated SHR.

In conclusion, the central manipulation that promoted the decrease in the blood pressure values and sympathetic activity also affected the expression in the target organs, mainly through the up-regulation of angiotensinogen and AT-2 genes in the kidney and down-regulation of AT-1 in the heart.

Therefore, our data suggests that a decrease in SNS activity through reduction in the activity of sympatho-excitatory regions, can be one possible way to control BP and PVN and RVLM could constitute areas for novel therapeutic interventions to long-term control of BP and end-organ protection in AHT. However, it is important to consider that the balance between components of RAS is not linear; therefore any final functional result from modification of RAS components expressions can only be proved by comprehensive studies.

New findings and its importance under working hypothesis 3

Our main finding was that treatment of LVV-hKir2.1 promotes the remodeling process in target organs, as showed by the different regulation of gene expressions in cardiac and renal tissues of treated SHR. Among the 9 genes studied in the heart we did not found differences between the PVN treated SHR group and the WKY group. In the heart RVLM treated SHR, only one gene was downregulated – myosin 7 (3,6 fold) - in relation to WKY group. In relation to the sham SHR there was a downregulation of AT1 (3,9 fold and 3,8 fold), ATP2C1 (2,6 fold and 2,5 fold) and Tnnt2 (2,7 fold and 2,0 fold) in treated PVN SHR and in treated RVLM SHR, respectively. Also, Tpm2 was down-regulated (3,0 fold) in treated RVLM SHR.

In the kidney of treated PVN and RVLM SHR, between the 8 genes studied only 2 were up-regulated – endothelin 1 (3,0 fold and 5,3 fold) and AT2 (2,2 fold and 8,3 fold), respectively, in comparison with the WKY rats. Comparing the mRNA expression in treated SHR with sham SHR rats: 3 genes were up-regulated – Angiotensinogen (1,9 fold and 2,7 fold), AT2 (11,1 fold and 42,1 fold) and ET-1 (8,5 fold and 14,7 fold) in treated PVN SHR and in treated RVLM SHR, respectively.

In the carotid artery, in the PVN SHR, among the five genes studied only one was up-regulated (1,7 fold) - endothelin receptor type A (Ednra) – compared with WKY rats. Comparing the mRNA expression in treated PVN SHR with sham SHR rats 1 gene was a down-regulated - endothelin converting enzyme 1 (3,0 fold) - and 1 gene was up-regulated - endothelin-2 (ET-2) (4,8 fold). In RVLM SHR group, two were up-regulated – endothelin converting enzyme 1 (Ece1; 2,1 fold) and endothelin receptor type A (Ednra; 1,5 fold) - compared with WKY rats. Comparing with the sham SHR group there was an up-regulation of Ece1 (2,0 fold) and ET-2 (3,0 fold) in RVLM treated SHR.

Therefore, the central manipulation that promoted the decrease in the blood pressure values and sympathetic activity also affected the expression in the target organs, mainly through the up-regulation of angiotensinogen and AT-2 genes in the kidney and down-regulation of AT-1 in the heart. These results provide new insights into the molecular mechanisms underlying the potential efficacy of chronic overexpression of hKir2.1 channels in central sympathoexcitatory areas in protecting against end-organ damage in essential AHT and thus lay the basis for future mechanistic studies.

Submitted to Acta Physiologica

Reversal remodeling of blood pressure control genes following chronic depression of Paraventricular Nucleus of Hypothalamus and Rostroventrolateral Medulla in spontaneous hypertensive rats by V Geraldes, N Gonçalves-Rosa, R Laires, JF Paton, TF Outeiro and I Rocha

CHAPTER 4

CHAPTER 4

DISCUSSION

I. Discussion of the hypotheses under study

Several studies have pointed out that the persistent increase in sympathetic tone is a major contributor to both the initiation and maintenance of the hypertensive condition (Yamada et al., 1988; Grassi, 2004b; Smith et al., 2004; Guyenet, 2006; Fisher & Paton, 2012). In fact, increased sympathetic activity has been detected in normotensive individuals with a family history of hypertension and in individuals with essential hypertension but not in those with secondary hypertension (Yamada et al., 1988; Grassi et al., 1998; Grassi, 2004a, 2009).

Likewise, high plasmatic nor-epinephrine levels have also been associated with essential hypertension being consistently increased in younger hypertensive patients (Grassi, 1998) and increased peripheral sympathetic nervous activity has been detected by microneurography techniques in patients with hypertension (Anderson et al., 1989; Grassi, 1998; Greenwood et al., 1999; Mano, 2012).

Therefore, it is well established, that augmented sympathetic nervous system (SNS) activity is related with hypertensive conditions. From experimental models of hypertension and hypertensive patients data using microneurography and norepinephrine spillover techniques, there is evidence that the sympathetic influence upon the cardiovascular system is often increased when blood pressure is chronically elevated. But, the precise mechanisms leading to sympathetic activation in essential hypertension remain to be elucidated despite having been suggested that the increased sympathetic activity is due to alterations of autonomic reflex pathways and/or in brain sites (Fisher *et al.*, 2009).

In the present work, we investigated the effect of over expressing a potassium inwardly rectifying channel in the PVN and in RVLM to lower their neuronal activity examining its consequences upon long term blood pressure regulation in an animal model of hypertension. For that, a human inward rectifying potassium channel (hKir2.1) was over-

expressed under the control of a synapsin promoter, that was neurone specific (Duale et al., 2005a; Duale et al., 2005b).

Results show that chronic overexpression of potassium channels in the PVN and RVLM of conscious unrestrained SHR caused a marked and sustained decrease in blood pressure and sympathetic output as revealed indirectly by a decrease in the power density of the Low frequency (LF) band of systolic blood pressure (SBP). In the PVN, in particular, there is a reversal remodeling of the baro- and chemoreceptor function that approached the normal physiological function. Interestingly, no changes in the baro- and chemoreceptor function were observed with intervention in RVLM, where the sympathetic efferent response is primarily generated. Signalling changes also occurred in hypertensive target organs, heart, kidney and vessels. In fact, the central manipulation of neuronal cells excitability that promoted a decrease in blood pressure and sympathetic activity also affected gene and molecular expression in hypertensive target organs, mainly through the up-regulation of angiotensinogen and AT-2 genes in the kidney and down-regulation of AT-1 receptors in the heart.

Effects on arterial blood pressure: A strong decrease of blood pressure, systolic (26 mmHg, 39 mmHg), diastolic (22 mmHg, 40 mmHg) and mean BP (22 mmHg; 40 mmHg) was observed after the modulation of cells excitability in PVN and RVLM, respectively at 60 days post-microinjection. This decline of SBP and, which were accompanied by a decrease in HR, was statistically confirmed at 30 and 40 days after the lentiviral microinjection on both regions, and persisted until the animals were humanely sacrificed. Sham rats did not show decreases in BP during the recorded period. In contrast to the RVLM, LVV-hKir2.1 injection in the MCPA didn't evoke any changes on blood pressure and heart rate over the same time frame. This different behavior of MCPA might be explained by the fact that the MCPA neurons do not depend on the integrity of the RVLM or suprabulbar regions as described before (Seyedabadi *et al*., 2006).

MCPA consists in a group of neurons in cervical spinal cord white matter located in the ventrolateral region of the medullo-cervical junction, from the most caudal levels of the medulla into the upper cervical spinal cord thus. These neurons project to the spinal cord and pregnaglionic sympathetic neurons. Despite, when stimulated large pressor responses are evoked, its role on cardiovascular regulation is not yet determined. In our

study, MCPA was used as a control sympathoexcitatory area due to the apparent lack of neuronal relay to the RVLM and PVN. In this way, our results may suggest that not all the sympathoexcitatory areas, even if they to evoke changes on blood pressure and/or affect preganglionic sympathetic neurons tone, intervene on the sympathoexcitation observed in hypertension. This assumption is in line with observations in previous studies of our lab, which revealed that lentiviral injection at periacqueductal gray matter (PAG) was unable to induce blood pressure and heart rate changes despite this area coordinates specific patterns of cardiovascular modulatory responses related to stressful stimulus. Due to its anatomical location interfacing the forebrain and the lower brainstem, PAG, in opposition to MCPA, receives selective inputs from the prefrontal cortex, amygdala, hypothalamus and nociceptive pathways and has neuronal connections with several brainstem nucleus involved in the generation of behavior specific patterns of motor and autonomic responses.

Is SHR the best animal model for studying neurogenic hypertension?

The animal model used in this study was the spontaneously hypertensive rat (SHR). In the SHR, the contribution of SNS to the maintenance of elevated blood pressure values is described by several authors (Yamori et al., 1969; Aoki et al., 1973; Judy et al., 1979; Webb et al., 1981; Abboud, 1982; Folkow, 1982; Smith et al., 1984; Simms et al., 2009; Geraldes et al., 2013). Also, in this animal model, the sympathetic activity is known to be over-activated even before hypertension develops (Simms et al., 2009). Previous studies suggested that in SHR there is an increased excitatory drive from PVN and RVLM neurons that is associated with an elevated sympathetic outflow (Allen, 2002, Bergamaschi et al., 1995; Ito et al., 2000, 2001; Ito et al., 2002; Ito et al., 2003).

On the choice of the target regions of PVN, RVLM and MPCA

Although several regions of the central nervous system contribute to sympathetic tone, we chose these two regions, since the RVLM is a major source of sympathetic activity (Ross et al., 1984; Dampney, 1994; AM, 2001) and the PVN is well known for its importance in autonomic control and, in particular, for cardiovascular regulation. As a

control area was used the medullo-cervical pressor area (MCPA) located in the ventrolateral region of the medullo-cervical junction.

The PVN neurones project either directly to the spinal cord or to the RVLM (Coote, 2007) thereby accessing sympathetic neurones to modulate blood pressure (Hosoya et al., 1991; Loewy, 1991; Coote, 1995; Ranson et al., 1998; Motawei et al., 1999; Pyner & Coote, 1999, 2000; Badoer, 2001; Coote, 2005).

The extensive projections of the PVN to central regions (RVLM, area postrema, NTS and intermediolateral nucleus of the spinal cord) indicate that PVN plays a significant role in modulating RVLM activity and sympathetic outflow. The PVN receives input from a large number of regions in the brain, including those associated with osmotic control, appetite and energy metabolism, stress and other areas that exert effects on BP. Thus, it is clear that the role of the PVN is to integrate inputs from a variety of sources and modify RVLM activity according (Aiyagari et al., 2011).

Electrolytic lesions of the PVN in SHR elicited an acute reduction of sympathetic activity together with a decrease of blood pressure (Takeda et al., 1991). Other acute studies, performed under general anesthesia, showed that PVN muscimol injections lowered BP and renal sympathetic nerve activity both in SHR and WKY rats, indicating that this region was tonically active in both animal strains to control BP and peripheral sympathetic activity (Allen, 2002).

RVLM is an important sympatho-excitatory region that plays a key role in controlling peripheral sympathetic nerve activity and blood pressure and in mediating baroreflex sympatho-inhibition (Loewy & Spyer, 1990a; Chalmers & Pilowsky, 1991; Dampney, 1994; Bergamaschi *et al.*, 1995; Ito *et al.*, 2000, 2001). In fact, the RVLM is the final major brain region that controls sympathetic nervous system activity, since it contains motor neurons that provide tonic drive to the spinal cord preganglionic motor neurons that directly regulate SNS activity (Izzo *et al.*, 2008).

Specific activation of RVLM neurons causes an increase in arterial blood pressure mediated by an increase in total peripheral resistance, cardiac output, and secretion of catecholamine's (Feldberg & Guertzenstein, 1972; Campos Júnior & Guertzenstein, 1989; Colombari *et al.*, 2001). Previous studies suggested that in SHR there is an increased

descending excitatory drive from RVLM neurons that is associated with an elevated sympathetic outflow (Bergamaschi *et al.*, 1995; Ito *et al.*, 2000, 2001; Ito *et al.*, 2002; Ito *et al.*, 2003). Most recently, part of the enhanced RVLM activity was shown to be of pre-synaptic origin and based on elevated synaptic drives from pre-inspiratory and post-inspiratory neurons (Moraes *et al.*, 2014).

Moreover, the increased activity of PVN and RVLM neurons are associated with the maintenance of high blood pressure values (Matsuura et al., 2002; Guyenet, 2006; Nassar et al., 2011; Kumagai et al., 2012; Moraes et al., 2014). Therefore, the relation of PVN and RVLM neurons to sympathetic control suggests that the spontaneous discharge can be modified through either changes to the intrinsic rate of depolarization or alterations in the balance of excitatory and inhibitory afferent input (Carlson & Wyss, 2011).

The MCPA is another sympathoexcitatory region that is located in the most ventrolateral medulla that extends caudally as far as the third cervical segment. This pressor area is distinct from the caudal pressor area (CPA), because is not dependent on the integrity of the RVLM and does not appear to mediate its effects via suprabulbar regions but via bulbospinal sympathetic neurons in the region. Using retrograde tracing MCPA neurons projecting to thoracic levels (which are neurochemically heterogeneous) that directly innervate the sympathetic preganglionic neurons (Seyedabadi *et al.*, 2006). Studies have demonstrated that bilateral RVLM blockade eliminates the responsiveness of the more rostrally located CPA (Gordon & McCann, 1988; Possas *et al.*, 1994; Natarajan & Morrison, 2000). In contrast, responses evoked from the MCPA are unaffected by bilateral RVLM blockade (Seyedabadi *et al.*, 2006). Thus, it seems that the MCPA does not appear to play a role in maintaining vasomotor tone after RVLM blockade and is distinct in both location and axonal outputs to the CPA.

Several studies, both in human subjects and animal models, have demonstrated an association between the circadian variation of BP values, the hypertensive condition, the sympathetic activation, the end-organ damage and the worsening of cardiovascular outcome (White, 2000; Pickering & Kario, 2001; Weber, 2002).

Thus, the idea of a long-term modulation of the level of sympathetic activity, at its central origin, as a way to control, and treat, high blood pressure, increasing cardiovascular compliance and protecting against end-organ damage is very appealing. In particular, the

manipulation of sympathetic cell excitability by modulation one of K+ channel expression, to hyperpolarize neuronal resting membrane potential, is an attractive hypothetical therapeutic strategy (Duale et al., 2007).

On the choice of the lentiviral factor

Lentivirus was used as its expression has been shown sustained within PVN neurones long term (Coleman et al., 2003). In previous studies, Duale et al (2007) and Howorth et al (2009) showed that hKir2.1 over-expression hyperpolarized the membrane potential of cultured cathecolaminergic PC12 cells by ~10mV which is expected to "electrically silence" neurones (Duale et al., 2007; Howorth et al., 2009). Similar over-expression strategies have been used to reveal that electrical silencing of neurones affecting development in ovo (Yoon et al., 2008) neuronal activity in vivo (Okada & Matsuda, 2008) and the ability of neurones to make and maintain connections in vivo (Yu et al., 2004; Mizuno et al., 2007; Hendy, 2010). This viral mediated approach has the advantage of being site specific and enabling over-expression in adulthood, avoiding the development of putative compensatory mechanisms associated with transgenic animals (Hendy, 2010).

On the blood pressure and autonomic output data

It seems that the RVLM treatment, takes longer to produce the effect in SBP, but generates a higher drop in blood pressure when compared with treatment in the PVN. On the other hand, DBP changes were only significant after 50 days in the PVN and 30 days in RVLM, suggesting that the LVV-treatment according to the injection site involves different mechanisms. In PVN, first promotes a decrease in SBP (at 30 days) and then a decrease in DBP (at 50 days). In RVLM, the fall in BP occur first in DBP (at 30 days) and then in SBP (at 40 days).

Interestingly, in the PVN, the decrease in LF spectra of SBP (indicative of sympatho-inhibition) occurred before the fall in SBP (i.e. 20 versus 30 days) suggesting a putative association between the changes on both variables. The same was not observed in RVLM.

Further, the fall in HF SBP, that occurred in both areas is indicative of reduced respiratory modulation of arterial pressure and could include reduced respiratory-sympathetic

coupling, a phenomena known to raise total peripheral resistance in the SHR (Simms et al., 2009).

On the baro and chemoreceptor function in PVN

In PVN, the effect in blood pressure and sympathetic output could be explained by the associated improvement of baroreflex gain and/or a down-regulation of peripheral chemoreflex responsiveness to stabilize lowers levels of blood pressure, as we observed. We propose that these changes were a result of reduced electrical excitability of PVN pre-motor sympathetic neurones but cannot rule out reduced release of vasopressin and oxytocin. This is consistent with our neuroanatomical western blot analysis confirming hKir2.1 protein over-expression was within the PVN region.

In fact, it is well accepted that neurogenic hypertension is accompanied by an impairment of the baroreceptor reflex (Grassi et al., 1998). Our data showed that depressing PVN neuronal activity improved baroreflex gain. Previous work from several authors has shown that during the course of an alerting reaction there is a decrease in baroreflex efficacy and a facilitation of the carotid chemoreceptor reflex due to modifications of synaptic integration at the level of the NTS; this might include mechanisms involving GABA and angiotensin II release within the nucleus tractus solitaries (Jordan et al., 1988; Spyer, 1990; Silva-Carvalho et al., 1995a; Silva-Carvalho et al., 1995b; Kasparov et al., 1998; Kasparov & Paton, 1999; Head & Mayorov, 2001; Rocha et al., 2003). Such an angiotensinogenic mechanism seems to be particularly active in pathophysiological conditions like myocardial ischemia and hypertension (Rocha et al., 2003; Rosário et al., 2003; Maximino et al., 2006) and its behavior can be modulated by intervening pharmacologically on NTS AT1 receptors (Kasparov et al., 1998; Kasparov & Paton, 1999; Rocha et al., 2003; Rosário et al., 2003). In fact, during myocardial ischemia, AT1 blockade reversed the remodeling of baroreceptor and chemoreceptor reflex function in a way similar to that elicited upon the over-expression of hKir2.1 in PVN neuronal cells (Rocha et al., 2003; Rosário et al., 2003).

On the baro and chemoreceptor function in RVLM

Despite the fall in arterial pressure in the SHR, LVV-hKir2.1 microinjection had no effect on the peripheral chemoreflex evoked cardiovascular and respiratory responses. This result was unexpected given the importance of the RVLM in mediating the peripheral chemoreceptor reflex evoked sympathoexcitation in acute anaesthetized rats (Koshiya & Guyenet, 1996a). We can hypothesize that the chronic depression of RVLM excitability could lead to neuronal plasticity and enhanced functional expression of peripheral chemoreflex pathways that bypass RVLM, such as those routing via the PVN, the lateral hypothalamus or the pre-limbic cortex (Owens & Verberne, 1996; Olivan *et al.*, 2001; Gabbott *et al.*, 2005).

In the RVLM the microinjection of the lentiviral vector (LVV-hKir2.1) did not evoke any change in the baroreflex sensitivity (BRS) in the SHR. This is already impaired in the SHR compared to normotensive rats, indicating a deficit in the vagal capacity to reduce heart rate (Verberne *et al.*, 1988; Widdop *et al.*, 1990; Minami & Head, 1993). However, impairment of BRS controlling heart rate was not associated with impairment of BRS controlling efferent sympathetic nerve activity in human hypertension (Grassi *et al.*, 1998) suggesting distinct reflex pathways. So, the impairment of BRS in the SHR is related to the efferent parasympathetic, vagal pathway. Due to these facts, we were not surprised that there are no changes in baroreflex gain, since this reflects cardiac reflex gain that is mainly determined by the activity of cardiac vagal motoneurons and these were not targeted in the present study.

Given that the peripheral chemoreflex and the baroreflex were tested under anesthesia the depressant effect of the agent cannot be neglected as it may exacerbate the reduced excitability of RVLM neurons and might alter the normal pattern of cardiovascular control (Korner, 1971). In particular, by stimulating the GABA-ergic system barbiturates will enhances the inhibitory pathway between the caudal ventrolateral medulla and the RVLM, further decreasing RVLM excitability. Future studies should focus on the baroreflex sympathetic vasomotor gain in SHR before and after LVV-hKir2.1 in the RVLM.

On the respiratory data

Interestingly, respiratory rate remained unchanged in all experimental groups suggesting that there is no tonic excitatory drive from the PVN or RVLM affecting this variable in hypertensive rats. Additionally, we see no tonic influence from the PVN on the resting arterial pressure level in normotensive rats, which contrasts with a previous acute in vivo study (Allen 2002).

On the day and night data

The demonstration of a non-dipper blood pressure profile in animal models remains difficult mainly due to the failure in establishing a clear distinction between day and night values. This was confirmed in our study as through PVN or RVLM-sympathetic manipulations, we were only able to modify BP light/dark values of SHR which approached those of WKY rats. However, we were unable to modify the day and night profile of BP values variation in both strains. This inability of defining a light/dark profile in rats similar to the one set for human subjects may be due to rats intermittent behavior alternating awake and sleep periods both in the light and dark phase. Probably the only way of better defining rats light and dark phase profiles would be by monitoring of cerebral activity through EEG which was out of the scope of the present work.

Interestingly, LVV-hKir2.1microinjection of SHRs in RVLM promoted the concurrent overall decrease of LF power of systolic blood pressure with the lowering of HR in the light phase of the 24h period when the animals have a strong decrease in their activity with longer periods of sleep. Despite the study design was not appropriate to analyze the sleep awake cycle, the decrease of HR suggests a regained circadian variation of HR and a rebuilt, at least partially, of the circadian clock. In fact, a slowing of the heart during sleep is well established despite some controversy on the origin of the circadian variation of HR, which seems more likely due to a sympathetic withdrawal rather than a parasympathetic effect (Massin et al., 2000).

However, Makino et al, reported, in contradiction to our results, that the impairment of the baroreceptor reflex is known to eliminate selectively the circadian rhythms of cardiovascular BP and that the elimination of the sympathetic nervous system suppresses

the circadian rhythms of BP and HR by decreasing BP and HR during the dark period (Makino et al., 1997). Further studies need to be performed to clarify the autonomic modulation upon circadian rhythms.

On signalling changes evoked by the decrease of sympathetic activity

Gene expression analysis is one of the several recent technologies available for studying cardiovascular signalling systems under physiological or pathological conditions. It is also a valuable tool to evaluate the molecular remodeling of tissues under therapeutics of different natures (pharmacological, surgical, devices). As mentioned before, the heart, kidney, vessels and brain are key hypertensive target organs. In fact, hypertension, specially non-treated hypertension, accelerates these organs damage in such a way that it can provoke eventual organ failure and cardiovascular disability or death. In the moment, there are some pharmacological schemes that not only decrease blood pressure values but also interfere with the damaging processing leading to a reverse remodeling process.

In order to determine the existence and the direction of the signalling changes by the decrease of sympathetic activity induced by chronic overexpression of hKir2.1 channels in the PVN and RVLM, the mRNA levels of 17 pre-selected genes have been evaluated. Our overall finding is that the decrease of sympathetic activity induced a remodeling process in molecular and genetic signalling at the target organs.

a) Heart and vessel

Taking in to account the 9 genes evaluated in the present study, only 1 was different from WKY rats – the myosin heavy chain β (β-MHC). The β-MHC 7 (V3 cardiac myosin) was downregulated (3,6 fold) in treated RVLM SHR and are involved in regulation of contractility or hypertrophy. Compagno showed that the β-MHC was markedly expressed in the ventricle of 15-week old SHR vs WKY (Compagno *et al.*, 2001). Hence, our results showed that the LVV-treatment decreased the β-MHC expression in RVLM SHR in comparison to the WKY rats, similar to Ang II receptor type 1 antagonist that promotes the decrease in BP and downregulate MHC in the aorta of SHR (Fujii *et al.*, 1999).

There was no significant difference between treated SHR and WKY rats in the expression of AT1 receptor, ATPase Ca^{2+}, Endothelin-1, Myosin 6, Nitric oxide synthase 3, Troponin T2, Tropomyosin 1 (α) and Tropomyosin 2 (β) in the heart.

Both treated SHR had a down-regulation of AT1 (3,9 fold and 3,8 fold), ATP2C1 (2,6 fold and 2,5 fold) and Tnnt2 (2,7 fold and 2,0 fold) in the heart. The RVLM SHR group has also showed a down-regulation of Tpm2 when compared to the SHR sham group. Thus, it seems that there is a remodeling process in the hypertrophied heart of this animal model, since there is a continued improvement in gene expression in the heart of these animals, approaching the normality. However in our study we did not include a measurement of wall stress, which is influenced by pressure, chamber radius and wall thickness.

In the carotid artery, there is an up-regulation of endothelin receptor type A (Ednra) in treated PVN and RVLM SHR in comparison to WKY rats (1,7 fold and 1,5 fold, respectively). We have also found an up-regulation (2,1 fold) of endothelin converting enzyme 1 (Ece1) - in treated RVLM SHR compared with WKY rats.

When compared to the sham group, the treated PVN and RVLM SHR showed an up-regulation (4,7 fold and 3,0 fold, respectively) of endothelin-2 (ET-2). In the PVN SHR group there is also a down-regulation of Ece1 (3 fold) and in the RVLM SHR group there is an up-regulation of Ece1 (2,0 fold). So it seems that the vasculature is trying to compensate the decrease in blood pressure values obtained by central manipulation though the up-regulation of Edrna (in the PVN and RVLM SHR group) and Ece1 (in the RVLM SHR group) .

b) Kidney

In the kidneys of PVN and RVLM treated SHR for the 8 genes studied only 2 were up-regulated -ET-1 and AT2 - with a fold change of 3,0 and 2,2 for PVN SHR and 5,3 fold and 8,3 fold for RVLM SHR, respectively, when compared to WKY rats. In the rest of the genes analyzed there were no differences between the two groups: Angiotensinogen, AT1a, AT1b, ATPase Ca^{2+}, Nitric oxide synthase 3 (Nos3) and Renin.

ET-1 is a potent vasoconstrictor but the role of ET-1 in SHR remains unclear. Hughes et al found that SHR and aged-matched WKY rats had no difference in renal ET-1 levels until AHT appeared. After the development of AHT the SHR had a significantly reduced ET-1 in

the urine and in the outer and inner medulla of the kidney (Hughes *et al.*, 1992; Largo *et al.*, 1997). Hence, the up-regulation of ET-1 found in the kidneys of treated SHR relative to normotensive rats (WKY) and to Sham SHR seems not to participate in the progression of AHT in this animal model.

The renin-angiotensin system in the kidney plays an important role in the regulation of hemodynamic and tubular functions and it is established that renin, angiotensinogen, and angiotensin converting enzyme can produce angiotensin II locally (Johns, 1989). Wu observed in the kidneys of SHR an up-regulation in Ang II receptor AT-1 in comparison to WKY rats (Wu *et al.*, 1994). Therefore, we can assume that the LVV-treatment in SHR can reduce the mRNA levels of AT-1 in the kidneys, since these are similar to the mRNA AT-1 levels in WKY rats, thus close to normality, which is also a limitation to the vasoconstrictive effect of Ang II.

Ang-(1–7) has been shown to directly downregulate another RAS component, the AT-1 receptor, in cultured vascular smooth muscle cells (Clark *et al.*, 2001). Our results showed an up-regulation in AT-2 receptor in the treated SHR, thus contradicting the biological effects of AT1 receptor activation, promoting vasodilation, growth inhibition and cell differentiation (Suzanne Oparil, 2003). In the present study we didn´t analyze the Ang-(1-7) expression, so, we can not speculate about the vasodilatation action of Ang-(1-7).

Cosentino *et al.* showed that long-term treatment with the AT1R antagonist, losartan, in SHR promotes a significantly increase in AT2R mRNA in thoracic aortas, supporting our finding about the beneficial up-regulation of AT2R mRNA in the kidney of treated SHR (Cosentino *et al.*, 2005).

An increased expression of AT2 by LVV-treatment may be a possible way to normalize or improve the peripheral chemosensitivity and result in decrease of sympathetic activation and blocd pressure in SHR (Geraldes *et al.*, 2013). In patients with systemic arterial hypertension, the statin therapy has the same effect though the down-regulation of AT-1 receptors. There are also studies showing the increase in NO synthase in SHR in comparison to WKY rats (Vaziri *et al.*, 1998; Fernández *et al.*, 2003), so it seems that the LVV-treatment tends to decrease NO synthase expression in the kidney of treated SHR similar to WKY rats.

In the kidney, the renin release is regulated by the renal sympathetic nerves (mediated by β1-adrenoceptors); by the pressure-sensitive renal baroreceptor; and by the macula densa, which is responsive to tubular fluid composition (Skøtt & Jensen, 1993). According to several studies, there is an increase in renin mRNA expression in the kidneys of SHR (Antonaccio *et al.*, 1984; Samani *et al.*, 1989; Nakamura & Johns, 1995). There is also evidence of a relation between renal sympathetic nerve activity and the production and release of renin. In fact, renal denervation in the rat blunted the increase in renal renin mRNA after long-term ureteral obstruction (el-Dahr et al., 1991). Similarly, renal renin mRNA levels were lower in denervated than innervated kidneys (Page et al., 1992). These studies showed that tonic activity in the renal nerves could elevate renin gene expression. Therefore, we can speculate that the decrease in sympathetic nerve activity promoted through the LVV-treatment can decrease the renin expression in the kidneys of treated SHR, since mRNA levels of renin are similar to WKY rats.

There is a positive correlation between the angiotensinogen levels and the blood pressure found in rats and in humans (Dzau VJ, 1989, El-Dahr SS, 1991, Page WV, 1992, Nakamura A, 1994; Bruna RD, 1993). Other studies using the rat and human angiotensinogen genes have suggested that the transcriptional mechanism of the angiotensinogen gene is involved in the pathogenesis of AHT (Ingelfinger *et al.*, 1986; Ingelfinger *et al.*, 1990; Nakamura & Johns, 1995). In addition, the angiotensinogen-deficient mice doesn´t produce angiotensinogen and are hypotensive, what shows the impact of angiotensinogen in the maintenance of BP and in the development of AHT (Pratt *et al.*, 1989; Tanimoto *et al.*, 1994).

The kidneys of the SHR contain lower levels of angiotensinogen mRNA compared with the WKY rats (Pratt *et al.*, 1989). There is also evidence that low levels of renal sympathetic activity may increase angiotensinogen gene expression (Nakamura & Johns, 1994). In our study, the LVV-treatment decreased the sympathetic activity and increased the angiotensinogen mRNA in the kidneys of treated PVN and RVLM SHR compared to sham SHR (1,9 fold and 2,7 fold) and to similar levels found in WKY rats. One hypothesis is that the decrease in sympathetic activity found after the LVV-treatment could be the cause for the increase in angiotensinogen gene expression found in the kidney of treated SHR.

Therefore, the central manipulation that promoted the decrease in the blood pressure values and sympathetic activity also affected the expression in the target organs, mainly through the up-regulation of angiotensinogen and AT-2 genes in the kidney and down-regulation of AT-1 in the heart. However, it is important to consider that the balance between components of Renin Angiotensin System (RAS) is not linear; therefore any final functional result from modification of RAS components expressions can only be proved by comprehensive studies.

On the concepts underlying sympathoexcitation in hypertension

Control of blood pressure requires complex integration of regulatory mechanisms across multiple physiological systems. A sustained increase in arterial pressure therefore reflects a failure of one or more of these controls.

One likely mechanism of essential hypertension is an increased in sympathetic nervous system. The increase of sympathetic outflow to the heart results in increased cardiac output and neurally mediated vasoconstriction leading to elevated blood pressure values (Schlaich *et al.*, 2012). So, the reduction of the enhanced sympathetic activity has been considered as an antihypertensive strategy (Del Colle *et al.*, 2007; Biaggioni, 2008; Signolet *et al.*, 2008; Fisher & Fadel, 2010; Grassi *et al.*, 2010). However, the precise mechanisms responsible for the sympathetic activation in essential hypertension remain enigmatic, since they are complex and multifactoral, although it is known that the interaction of genetic influences with behavioural and lifestyle factors are important. However several hypotheses can be discussed.

One hypothesis believed to be responsible for this sympathoexcitation is increased systemic and central angiotensin II signalling. Most of the Ang II actions are mediated by the angiotensin II type 1 (AT1) receptor and the central nervous system is richly endowed with AT1 receptors(Zucker & Gao, 2005). In fact, the paraventricular nucleus and the rostroventrolateral medulla appears to have an especially dense distribution of AT1 receptors and Ang II signalling is enhanced in this two central areas (Zucker & Gao, 2005). Plasma angiotensin II is increased in humans and animals with hypertension and exerts central sympathoexcitatory effects, promotes the release of norepinephrine and amplifies

the adrenoreceptor response to stimuli in subjects with elevated levels of renin and angiotensin, but also in subjects with low levels of rennin. Circulating angiotensin II also reduces the transmission between baroreceptor afferents and NTS efferent neurons by activating endothelial angiotensin II receptors type 1 (AT1). The mechanism of angiotensin II control of the baroreflex involves the production of nitric oxide (NO) by the capillary endothelium, and this mechanism could have a role in neurogenic hypertension (Paton *et al.*, 2001).

Another hypothesis is that the sympathetic hyperactivity may be due to insulin resistance, since the presence of hypertension is often associated with hyperinsulinemia and is known that insulin resistance/hyperinsulinemia increases the sympathetic traffic and the release of norepinephrine. However, the reciprocal is also true, so it is difficult to determine whether it is sympathoexcitation that precedes insulin resistance or otherwise. During short-term hyperinsulinemia, insulin-induced sympathoexcitation helps to maintain blood pressure, and not only sympathetic overactivation, but also denervation are associated with insulin resistance (Scherrer & Sartori, 1997). Moreover, there is evidence that suggests that insulin sympathoexcitatory effects are mediated at least in part by a central neural action (Muntzel *et al.*, 1995), since insulin crosses the blood-brain barrier (Margolis & Altszuler, 1967) and insulin receptors have been demonstrated in several distinct regions of the central nervous system (Sauter *et al.*, 1983).

Another mechanism is could be related to the fact that the sympathetic activation is associated with baroreflex dysregulation, since hypertension is characterized by a baroreflex modulation and sympathetic traffic resetting towards high blood pressure blood pressure values. The major objective of this mechanism is to maintain blood pressure, more than reduce the increased blood pressure values, since apparently also the action of other cardiac reflex arcs that affect the sympathetic outflow to the vessels, the release of norepinephrine and renin are inhibited.

Other hypothesis that is linked to the maintenance of increased central sympathetic outflow may be related to excessive subcortical control caused by persistent excessive environmental stress. Other hypotheses include adipokines, endothelial dysfunction, cyclic intermittent hypoxemia and aldosterone effects on central nervous system.

And the last hypothesis, which is the basis of the present work and has been previously described, is that the increased sympathetic activity may result from an abnormal elevated sympathetic drive from brain centres, such PVN and RVLM.

In conclusion, the present work shows that the intervention on central sympathoexcitatory neurone excitability through the genetic manipulation of potassium channels expression is able to alter peripheral blood pressure long term. This occurs by sympathetic outflow remodeling and by signalling changes that occurred in hypertensive target organs that maintain cardiovascular homeostasis. Our data, from an animal model, give insights into the pathophysiological mechanisms involved in the aetiology of essential hypertension of neurogenic origin and provide novel hypothetical therapeutic interventions at central level of the autonomic nervous system to control sympatoexcitation.

II. Summary of main results

This work focused on the enhanced sympathetic nervous system activity in an animal model of neurogenic hypertension. We found that:

1. The over expression of an inwardly rectifying potassium channel in the PVN and in the RVLM provided a long term (>60 days) anti-hypertensive response in conscious spontaneously hypertensive rats (SHR) that was associated with reductions in neurohumoral mediated vasoconstriction;

   a) LVV-hKir2.1 expression in PVN produced a time-dependent and significant decreases in systolic (158±3 to 132±6 mmHg $P<0.05$) and diastolic BP (135±4 to 113±5 mmHg $P<0.05$). SBP low frequency band was reduced (0.79±0.13 to 0.42±0.09 mmHg2; $P<0.05$), suggesting reduced sympathetic vasomotor tone.

   b) In RVLM, but not in MCPA, LVV-hKir2.1 expression produced a decrease in systolic (155±3 to 116±8 mmHg; SBP), and diastolic (130±3 to 90±12 mmHg) BP and HR (310±4 to 293±6) 60 days post-injection ($P<0.01$); Low frequency spectra of SBP decreased from 0.69±0.11 to 0.42±0.10 $mmHg^2$.

2. In the PVN, this decrease in blood pressure values was also associated with an enhanced baroreflex sensitivity and reduced peripheral chemosensitivity;
3. Blood pressure values and low frequency spectra of SBP in normotensive rats and in the sham groups (LVV-eGFP microinjected SHR) didn´t decreased;
4. Baro and chemoreflexes remain unchanged in in normotensive rats and in the sham groups;
5. Respiratory rate remained unchanged in all experimental groups;
6. LVV-hKir2.1 expression in PVN and RVLM produced a remodeling process in target organs:
    - **a)** In the heart among the 9 genes studied we did not found differences between the PVN treated SHR group and the WKY group. In the heart RVLM treated SHR, only 1 gene was downregulated – myosin 7 (3,6 fold) - in relation to WKY group.
    - **b)** In the heart, in relation to the sham SHR group there was a downregulation of AT1 (3,9 fold and 3,8 fold), ATP2C1 (2,6 fold and 2,5 fold) and Tnnt2 (2,7 fold and 2,0 fold) in treated PVN SHR and in treated RVLM SHR, respectively. Also, Tpm2 was down-regulated (3,0 fold) in treated RVLM SHR group.
    - **c)** There was no significant difference between treated SHR and WKY rats in the expression of AT1 receptor, ATPase Ca2+, Endothelin-1, Myosin 6, Nitric oxide synthase 3, Troponin T2, Tropomyosin 1 (α) and Tropomyosin 2 (β) in the heart.
    - **d)** In the kidney of PVN and RVLM treated SHR between the 8 genes studied only 2 were up-regulated – endothelin 1 (3,0 fold and 5,3 fold) and AT2 (2,2 fold and 8,3 fold), respectively, in comparison with the WKY rats.
    - **e)** In the kidney, comparing the mRNA expression in treated SHR with sham SHR rats: 3 genes were up-regulated – Angiotensinogen (1,9 fold and 2,7 fold), AT2 (11,1 fold and 42,1 fold) and ET-1 (8,5 fold and 14,7 fold) in treated PVN SHR and in treated RVLM SHR, respectively.
    - **f)** In the rest of the genes analyzed in the kidney there were no differences between the PVN and RVLM treated SHR group and the WKY group:

Angiotensinogen, AT1a, AT1b, ATPase Ca^{2+}, Nitric oxide synthase 3 (Nos3) and Renin.

g) In the carotid artery, in the PVN SHR group, among the 5 genes studied only 1 was up-regulated (1,7 fold) - endothelin receptor type A (Ednra) – compared with WKY rats. In RVLM SHR group, 2 were up-regulated – endothelin converting enzyme 1 (Ece1; 2,1 fold) and endothelin receptor type A (Ednra; 1,5 fold) - compared with WKY rats.

h) In the carotid artery, comparing the mRNA expression in treated PVN SHR with sham SHR rats 1 gene was a down-regulated - endothelin converting enzyme 1 (3,0 fold) - and 1 gene was up-regulated - endothelin-2 (ET-2) (4,8 fold). Comparing with the sham SHR group there was an up-regulation of Ece1 (2,0 fold) and ET-2 (3,0 fold) in RVLM treated SHR.

i) In the carotid artery, there was no significant difference in mRNA expression between treated SHR and WKY rats in: endothelin 1 (ET-1); endothelin 2 (ET-2) and endothelin receptor type B (Ednrb).

III. Strengths and limitations of the study

We propose that the changes found in sympathetic output and blood pressure values were a result of hyperpolarized PVN and RVLM pre-motor sympathetic neurons that leads to a reduced electrical excitability in that areas. However cannot rule out reduced release of vasopressin and oxytocin in the PVN.

The demonstration of a non-dipper blood pressure profile in animal models remains difficult mainly due to the failure in establishing a clear distinction between day and night values. This was confirmed in our study as through PVN-sympathetic manipulations, we were only able to modify BP light/dark values of SHR which approached those of WKY rats. However, we were unable to modify the day and night profile of BP values variation in both strains. This inability of defining a light/dark profile in rats similar to the one set for human subjects may be due to rats intermittent behavior alternating awake and sleep periods both in the light and dark phase. Probably the only way of better defining rats

light and dark phase profiles would be by monitoring of cerebral activity through EEG which was out of the scope of the present work.

In metabolic evaluation, the animals were not subjected to an adaptation period to the metabolic cages, which could impact on our metabolic data, constituting a study limitation.

The sympathetic activity was evaluated indirectly with power spectrum analysis of heart rate and blood pressure variability. Even though these indices are well validated for that purpose, it is important recognized that this is not a direct sympathetic drive evaluation and, as such, only association and not causation should be claimed.

Also, the fact that the study uses a rat model allows only conclusions/associations for mechanisms of hypertension in that model. Implications for treatment of hypertension should be addressed by subsequent human studies.

CHAPTER 5

CHAPTER 5

PERSPECTIVES AND FUTURE WORK

I. Possible mechanisms for sympathetic overactivity in hypertension

The genesis of the sympathetic nervous system overactivity found in pathological conditions, such hypertension continues to be an area of intense research.

In the central nervous system, the great majority of the key regulatory sites that control sympathetic outflow have already been identified despite some of their interrelationships are not yet completely defined. Beyond these physiological aspects, the challenge, now, is also to find a way to control the SNS or, in another words, to restore the SNS overactivity to *normal* levels.

The mechanisms contributing to central sympathetic activation are complex and multifactorial and involve several brain sites, neurotransmitters and neuromodulators (Weiss et al, 2003). It has been suggested that the increased sympathetic activity is due to alterations in autonomic reflex pathways and/or in the central autonomic neuroanatomical sites accompanied by modifications on hormonal and inflammatory factors.

Some hypotheses on sympathetic activity during hypertension have been advanced, such as the derangement of the sympathoinhibition exerted by the reflexogenic areas: arterial or cardiopulmonary baroreflexes, somatic reflexes and central or peripheral chemoreflexes that tonically could restrain adrenergic outflow (Brown & Fisher, 1980; Grassi *et al.*, 1997; Grassi, 2001; Lip *et al.*, 2007; Schultz & Li, 2007; Smith & Pacchia, 2007). Also, functional alterations in several autonomic areas including the RVLM, NTS and PVN that have been associated with changes in central concentrations of Ang II, aldosterone, NO, reactive oxygen species and inflammatory cytokines (Patel *et al.*, 2001; Zucker *et al.*, 2001; Zimmerman & Davisson, 2004; Gao *et al.*, 2005; Waki *et al.*, 2006; Yu *et al.*, 2007; Guggilam *et al.*, 2008; Zhang *et al.*, 2008; Waki *et al.*, 2010).

Another hypothesis, which has been tested and proven, is that sympathetic activation accompanying hypertension is in part due to an exaggerated central nervous system drive resulting from excessive environmental stress (Mancia *et al.*, 1997).

It has also been suggested that the metabolic alterations frequently detectable in hypertension, such as the hyperinsulinaemic state and the related insulin resistance, may be the triggering factors. This hypothesis is based on the evidence that insulin may have central sympathoexcitatory effect which may thus be enhanced the sympathetic tonus in hypertensive patients (Grassi *et al.*, 2007).

The sympathetic activation in hypertension can also depend on the renin–angiotensin system, since angiotensin II exerts central sympathoexcitatory effects and the pharmacologic blockade of the renin–angiotensin system via ACE-inhibitors or angiotensin II receptor blockers exerts sympathomodulatory effects (Fig. 5-1) (Grassi *et al.*, 1997; Grassi, 2001; Grassi *et al.*, 2007).

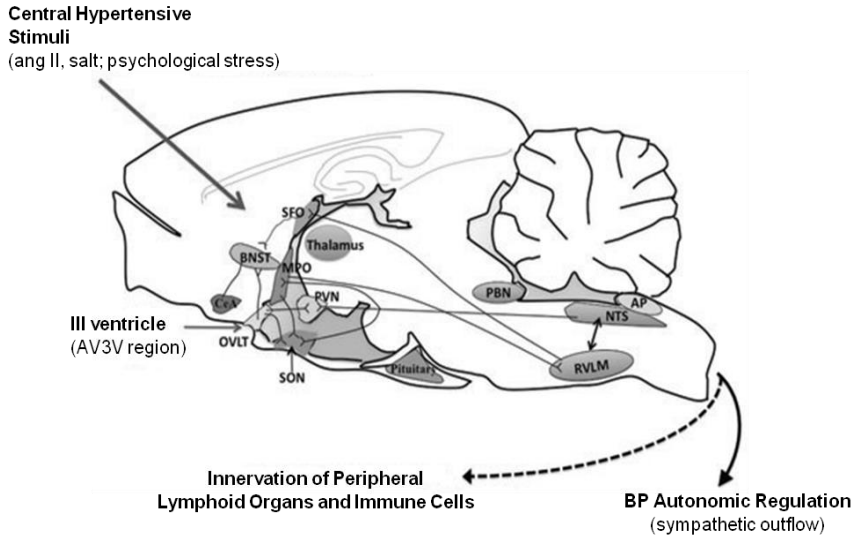


Figure 5-1. Diagram showing location and connections of some of the primary hypothalamic structures responsible for central angiotensin II signalling and the integration of the stress response. AP, area postrema; AV3V, anteroventral third ventricle; BNST, bed nucleus stria terminalis; CeA, central amygdala; MPO, median preoptic nucleus; NTS, nucleus of the solitary tract; PBN, parabrachial nucleus; PVN, paraventricular nucleus; RVLM, rostral ventrolateralmedulla; SFO, subfornical organ; and SON, supraoptic nucleus. Extracted from Marvar & Harrison, *Exp Physiol* 97.11 (2012).

Other studies indicate that increases in central sympathetic outflow can be mediated via Ang II activation of NAD(P)H oxidase with production of reactive oxygen species, which may directly activate central SNA pathways along with scavenging NO thereby removing the tonic restraint on sympathetic outflow (Fisher *et al.*, 2009).

Beyond the link between hypertension and angiotensin or reflexes impairment, essential hypertension may also be induced per se by changes in the cerebral vasculature leading to a decrease in the vessel internal diameter (Baumbach *et al.*, 1988; Baumbach & Heistad, 1988). This could be due to modifications of the shear stress upon the cerebral vessels walls (Agabiti-Rosei *et al.*, 2009). The induced vascular remodeling can be hypertropic in nature due to smooth cells increase in volume (Baumbach *et al.*, 1988) or eutrophic due to smooth cells novel rearrangements (Rizzoni *et al.*, 2009; Cates *et al.*, 2012). These narrowing of cerebral vasculature could activate primary a Cushing reflex that will, along time, be transformed in a persistent response, termed as the Cushing mechanism, which ultimately purpose is to improve brain perfusion. Interestingly, this progressive narrowing linked to a functional effect can be observed in young (pre-hypertensive) and adult SHR (Paton *et al.*, 2007; Paton & Waki, 2009).

In recent years, however, a major research effort has been focused on the role of inflammation in the genesis of hypertension. In fact, the role of brain inflammation in the pathogenesis of this disease is now a target of medical research and is the basis of our ongoing study (For detailed description, see II) (Paton & Waki, 2009; Felder, 2010; Marvar *et al.*, 2010; Shi *et al.*, 2010).

II. Future perspectives: the role of inflammation at PVN level in the origin of neurogenic hypertension

The idea that insufficient perfusion of key areas of the brain elicit an increase in sympathetic output and blood pressure due to the release of inflammation mediators is very attractive as a working hypothesis on the understanding of some of the mechanisms underlying neurogenic hypertension.

There is limited but compelling evidence that links essential hypertension with changes in cerebral circulation, inflammation and the adaptive immune system. When intracranial pressure is elevated >33mmHg over a short period, cerebral blood flow is significantly reduced. The elicited cerebral ischemia stimulates the vasomotor areas and systemic blood pressure rises, heart rate decreases and respiration is slowed accordingly.

This triad of reflex physiological responses helps to maintain cerebral blood flow and is termed the Cushing reflex. Not only Cushing but also Guyton and co-workers were impressed by the protective nature of this reflex response but they have just looked at it as the last effort of the brain to protect itself in ischemic conditions (Sagawa *et al.*, 1961; Smith & Guyton, 1963). In accordance, they speculated with others (Dickinson, 1991; Osborn, 2005) about the existence of a central baroreceptor system, located in the brainstem, which could be activated at normal blood pressure values.

In fact, the brainstem holds the primary station of sensory integration of autonomic nature as well the motor and respiratory centers. All of them participate on the generation of both baro and chemoreflex responses, the last one the major protective reflex of sympathetic nature.

Within the rostral ventrolateral medulla which projects neurons to the thoraco-lombar spinal cord, there are neurones which are intrinsically sensitive to hypoxia (Wang *et al.*, 2001) that can provide an explanation by which brainstem ischemia results in sympathoexcitation to ensure adequate central perfusion. The same type of neurons was found in the spinal cord (Braga *et al.*, 2007) showing that apparently the central nervous system is prepared to self protected itself to central ischemia and oxygen decrease, at different levels of the neuroaxis.

An association between narrowed vertebral arteries, due to atheroma plaques deposition and a medical history of essential hypertension was made back in the 1960's by Dickinson and Thompson. However, being this work performed post-mortem was not possible to understand which was the cause and the effect in this association.

But with time, as our understanding on end-organ damage associated with hypertension increased, the role of inflammation in these damaging processes as well as in the pathogenesis of hypertension have been highlighted.

The ATTICA study revealed that pre-hypertensive subjects have higher values of C-reactive protein, TNF-α, amyloid-a, homocysteine and white blood cell counts when compared with normal subjects and, authors suggested, that pre-hypertension might be a pro-inflammatory condition (Chrysohoou *et al.*, 2004). Other studies in hypertensive patients also showed increased levels of TNF-α, IL-6 and IL-1ß correlating positively with higher values of blood pressure (Dalekos *et al.*, 1997; Bautista *et al.*, 2005).

Studies in normal subjects indicated a putative link between plasma levels of ICAM-1 and IL6 and the values of systolic blood pressure, with the highest levels being related to systolic blood pressure values >140 mmHg (Chae *et al.*, 2001). Also, the administration of intracerebral angiotensin II, suggested as a major signal contributing to neurogenic hypertension (Brody, 1988; Johansson *et al.*, 1999; DiBona & Jones, 2001; Davern & Head, 2007), increased the genetic expression of proinflammatory splenic cytokines, such as interleukin-1β and interleukin-6 (Ganta *et al.*, 2005) together with an increase of blood–brain barrier permeability and cerebral microvasculature inflammation (Zhang *et al.*, 2010).

Also T-cells seem to play an important role in the development of hypertension as they can produce cytokines and release other mediators that affect the smooth muscle cells and vascular endothelium (Guzik *et al.*, 2007). The link between angiotensin II and T-cell activation has been showed by several authors. In an experimental animal model of T-cells lacking, mice failed to become hypertensive when were infused for 14 days with angiotensin II (Guzik *et al.*, 2007; Hoch *et al.*, 2009; Marvar *et al.*, 2010). These data indicate that not only angiotensin II facilitates the entrance in the brain of inflammatory cells and cytokines but also that central angiotensin II can elicit a peripheral immune

response via the autonomic nervous system as the autonomic denervation of the spleen abolished the immune response (Marvar *et al.*, 2010).

Microglial cells are also activated in hypertension, and their inhibition reduces blood pressure (Shi *et al.*, 2010). Thus, it can be hypothesized that pathological signalling molecules, most of them linked to neuroimmune mechanisms, released from the vasculature would exert a deleterious action on neuronal excitability, which in turn, would alter both the central set point of arterial pressure and its reflex control (Paton & Waki, 2009; Waki *et al.*, 2010). In these conditions, the brain could be both the target for inflammatory mediators in hypertension and a mediator of inflammation through its communication with the immune system.

Several studies showed that pro-inflammatory chemotactic proteins are more produced in the brainstem areas involved in cardiovascular control of the spontaneously hypertensive rats (SHR) than in normotensive rats (Waki *et al.*, 2007; Waki *et al.*, 2008). Also, some drugs used to treat hypertension also have general anti-inflammatory effects in the brain (Zhou *et al.*, 2005; Benicky *et al.*, 2009).

In our work, we showed that the decrease of neuronal excitability in two central areas evoked a decreased of sympathetic activity and blood pressure. This work called the attention, for the first time, for RVLM role on hypertension but the morphological and functional reverse remodelling was more effective through PVN genetic manipulation. Accordingly, and despite several studies (Paton & Waki, 2009; Waki *et al.*, 2010; Waki *et al.*, 2011; Almado *et al.*, 2014) pointed the brainstem as the key area for the initiation of the sympathoexcitation and increase of blood pressure, we believe that the hypothalamus, and in particular the PVN is the central area to be target in hypertension of neurogenic origin (Hilton & Spyer, 1980; Spyer, 1989; Benarroch, 2006; Macefield *et al.*, 2013).

In fact, the control of visceral functions and adaptation is integrated throughout the areas of the central autonomic network. The hypothalamus, through all its subdivisions, is part of that network and has a central role on the integration of autonomic and endocrine responses critical for homeostasis and adaptation to internal and external stimuli of several origins.

Acute and transient challenges triggering successful adaptations produce a short duration response consisting in sympathoadrenal excitation with tachycardia, hypertension, increase in cardiac output, redistribution of blood flow to the limbs, inhibition of the baro- and facilitation of the chemoreflex (Hilton & Spyer, 1980; Spyer, 1989; Silva-Carvalho *et al.*, 1995a; Silva-Carvalho *et al.*, 1995b; Benarroch, 2005; Benarroch, 2006). However, if the stimuli persist, a persistent adaptative response of deleterious nature is elicited leading to "pathological adaptations" both at central and peripheral level (Cersosimo & Benarroch, 2013; Zucker *et al.*, 2014).

The hypothalamus also contains osmosensitive and Na^+ sensitive neurons critical for water and sodium homeostasis. Likewise is a site of bidirectional communication between the immune system and the autonomic and the endocrine systems (Rivest, 2001; Benarroch, 2006; Nater *et al.*, 2013), eg, is well documented that during febrile conditions, circulating cytokines stimulate prostaglandin E_2 secretion at hypothalamic levels, which results in activation of the hypothalamo-pituitary-adrenocortical and sympathetic outputs leading to an inhibition of inflammatory response (Rivest, 2001; Benarroch, 2005).

In particular, the PVN generates coordinate endocrine and autonomic responses to stimuli of different nature including vasopressin secretion, and activation of sympathetic, adrenomedullary and adrenocortical systems. Also the different neuronal clusters within this nucleus respond to visceral, limbic, and humoral signals such as pain, fear and circulatory cytokines (Hilton & Spyer, 1980; Spyer, 1989; Stern, 2001; Benarroch, 2005; Kc *et al.*, 2010; Japundžić-Žigon, 2013; Hueston & Deak, 2014; Ni *et al.*, 2014).

Measurements of hypothalamic gene expression together with electrophysiological studies suggest that AT1 receptors, in the PVN, mediate inflammation (de Kloet *et al.*, 2013). This central inflammation is associated with metabolic and cardiovascular disorders and the deletion of PVN AT1 receptors decreases the levels of inflammation in this central area (de Kloet *et al.*, 2013).

The SHR, a widely used as an animal model of hypertension, also presents an activated inflammatory system, in a similar way to what seen in human hypertensive patients. Overall, the increased inflammation observed in both, SHR and patients with essential hypertension, may precede the onset of hypertension.

It is also believed that this inflammatory process may contribute to end-organ damage observed in the SHR (Schmid-Schönbein *et al.*, 1991). Results of several genetic expression studies revealed the supportive role of the hypothalamus and brainstem for factors such as neuronal nitric oxide synthase, inflammation and reactive oxygen species and their influence on hypertension of neurogenic origin (see (Marques & Morris, 2012), for a review).

Accordingly, and as an attempt to contribute to the future clarification on the role of PVN and inflammation in the origin of neurogenic hypertension, we started a new set of protocols intended to understand the role of central and localized inflammation on the genesis of the higher sympathetic tone and hypertension.

For that, a model of central inflammation in rodents by the stereotaxic administration of the Escherichia coli wall lipopolysaccharide (LPS) (Espinosa-Oliva *et al.*, 2013) was used. This model postulates that glial cells are activated after intracranial microinjection of LPS, inducing cytokines and chemokines production in the brain (Szczepanik *et al.*, 1996; Sun *et al.*, 2008; Campbell *et al.*, 2012) together with an intense recruitment of monocytes (Montero-Menei *et al.*, 1994; Montero-Menei *et al.*, 1996).

Briefly, normotensive Wistar-Kyoto, male rats of 12 weeks old were used in this preliminary study. Blood pressure, heart rate and respiration were monitored by radiotelemetry. A craniotomy was performed using our previously determined coordinates for PVN and animals (n=6) were bilaterally microinjected with Escherichia coli serotype 055:B5 lipopolysaccharide (50ηL, 1mg/kg) into the PVN. Control rats were microinjected in the same region with sodium cloride (n=6).

At 20 days, animals were re-anesthetised and the trachea was cannulated to include tracheal pressure to set the previous recorded variables. Baroreceptor (phenylefrine) and peripheral chemoreceptor (lobeline) reflexes were stimulated twice with an interval of 5 minutes between each stimulation. Animals were killed with an overdosis of anesthetic and the brain was removed for histology.

Immunohistochemistry for microglial marker, laminin, VEGFR1, VEGFR2, PDGFR beta, IL-6 and lipocalin-2 was performed and the fluorescence intensity shown was related to expression of protein levels in each sample and measured with a semiquantitative

method. Blood pressure, heart rate, respiration and were evaluated. BP and HR variability and baroreflex evaluation were performed with on the frequency domain.

The very preliminary results show that during 60 days of radiotelemetry recording there were no changes on blood pressure (from 112±1 mmHg to 110±1 mmHg) or heart rate (from 378±4 bpm to 361±4 bpm) values. Also, no changes were found in autonomic evaluation. Immunofluorescence analysis will be done in order to confirm neuroinflammation in the PVN (*Ongoing work*).

APPENDIX 1

APPENDIX 1

I. The autonomic nervous system

Almost all bodily functions are dependent of the autonomic nervous system. The precision and biological importance of the autonomic nervous system (ANS) on the control of visceral functions is well known but the mechanisms by which ANS exerts these functions are not generally understood. Also, most of them are not yet completely known due to the complexity of the system and to its continuous adaptations that are complemented with the actions of the endocrine and somatomotor systems. However, these three systems are able to regulate individual's homeostasis, including the adaptations to internal and external stimuli (Fig. 1-A).

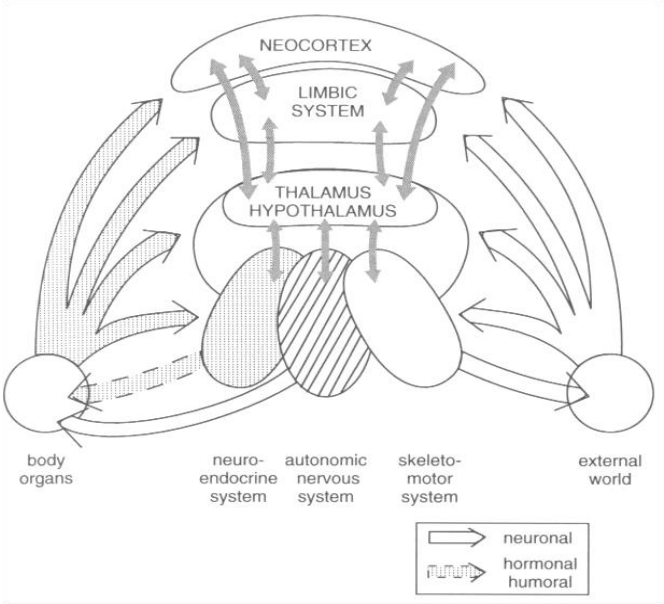


Figure 1-A. The interactions between the autonomic nervous system, the brain and the body. From Jänig, 2008.

The essential role of the autonomic nervous system is to integrate homeostatic and allostatic programs to distribute specific signals generated in the central nervous system, either in the resting state or during particular body behaviors, to the various target organs.

Despite being a system that is able to hide its own dysfunction, the latter can occur and be perceived in three conditions: when there is a functional failure, a physical defect in

the nervous network and during the aging process. In this case, the term disautonomy or autonomic failure is used. In these conditions, the system remains overactivated and an allostatic load is maintained being believed to contribute to several diseases eg, hypertension, atrial fibrillation, myocardial infarction, obesity, diabetes, atherosclerosis, sleep apnea and metabolic syndrome (Dampney, 1994; Robertson & Biaggioni, 1994; Appenzeller *et al.*, 2000; Folkow, 2000; McEwen, 2000; McEwen & Wingfield, 2003; Rocha *et al.*, 2003; Rocha *et al.*, 2004; Jänig, 2008; Low & Benarroch, 2008; Oliveira *et al.*, 2009; Oliveira *et al.*, 2010; Mathias & Bannister, 2012; Geraldes *et al.*, 2014).

In the classical sense, ANS is one of two major divisions of the peripheral nervous system and works mainly through negative feedback mechanisms and under the concept of reflex arc, which is the basic morpho-functional unit of the nervous system. In this way, the system is able to have specific neuronal pathways in the periphery and specific organization in the central nervous system in order to have precision and flexibility in its actions. This implies that there is a common central integration, yet formed by various central areas, that have multiple but distinct peripheral motor pathways. The autonomic effector organs are diverse, and thus their cell types, making clear that the autonomic nervous system outweighs the other systems in size and diversity of its functions (Loewy & Spyer, 1990; Dampney, 1994; Mathias & Bannister, 2012; Jänig, 2008, Appenzeller et al, 2000).

Definitions

The usage of inappropriate terms when referring to the autonomic nervous system, lead to generalizations and scientific inferences, which are not in accordance with the neurobiology and differentiation of autonomic function, thus creating functional misunderstandings and wrong impressions on how the system works. Langley (1921) proposed the term autonomic nervous system to describe the peripheral nervous system that regulates body tissues and organs except the skeletal muscle. In the present text, we will use Langley neuroanatomical terminology and the terms sympathetic, parasympathetic and enteric only to refer to the motor portion of the autonomic reflex arc. This arc also includes integrative centers located at the central nervous system – central autonomic network- where is conveyed sensory information from peripheral sensors located at specific reflexogenic areas (Fig 2-A).

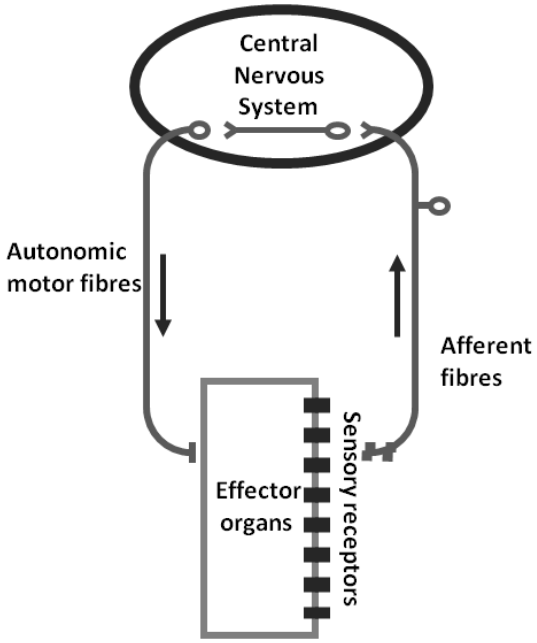


Figure 2-A. The autonomic reflex arc. The relative morphological relations between its different components are shown. The autonomic motor fibres include the sympathetic, parasympathetic and enteric divisions. From Rocha 2009.

The visceral afferent pathways

The afferent pathways are the interface between the visceral organs and the central nervous system. Most of the afferent fibres are unmyelinated but also myelinated fibres which can also conduct information up to about 30m/s (Loewy and Spyer, 1990). There are two types of visceral afferents: the primary afferent fibres and enteric afferent fibres, with the latter encoding chemical and mechanical events and having the cells bodies in the wall of the gastrointestinal tract (Loewy & Spyer, 1990; Jänig, 2008).

The nervous impulses of the primary afferent fibres are carried orthodromically to the spinal cord, brain stem or prevertebral sympathetic ganglia (Fig. 3-A) (Loewy & Spyer, 1990). The degree of physiological specificity of these afferent neurons is described by the elicited quantitative responses either to their chemical and mechanical stimulation.

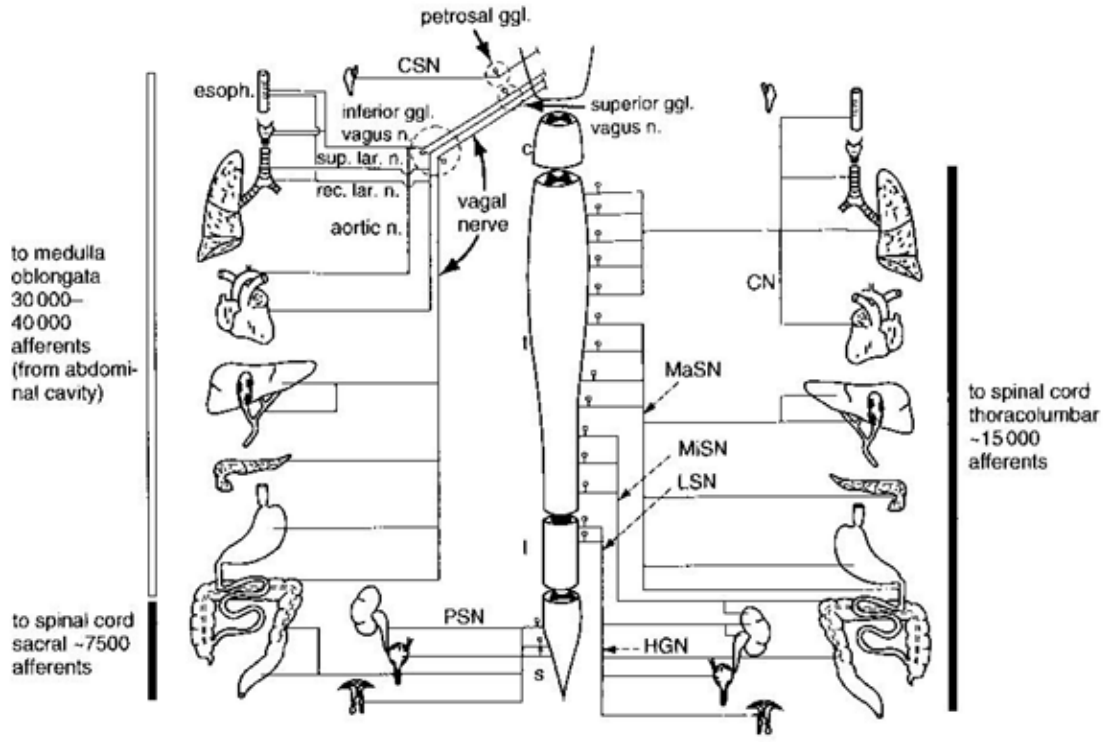


Figure 3-A. Projection of visceral afferent neurons. On the right side are shown spinal visceral afferent neurons projecting to the thoracic and upper lombar spinal cord whereas vagal afferent neurons projecting to the MTS and spinal visceral neurons projecting via splanchnic nerves to the sacral spinal cord. From Jänig, 2008.

The afferent neurons are involved in two main functions: the regulation of visceral actions including protective organ reflexes and the transport of painful information including pain from deep somatic tissues, hyperalgesia, deep pain and inflammation. This duality of function makes them fundamentally different from the somatic afferents, since the sensory and regulatory properties of the latter ones cannot be separated. In fact, the afferent impulses from skin or from muscle trigger a reflex and behavioural regulation as well as evoke a sensory experience, which is not true for the visceral afferents as some of their stimuli never reach the level of consciousness (eg, BP changes or gut distension) (Loewy and Spyer, 1990).

The primary afferents have their cells bodies in the spinal and cranial ganglia and their receptors are located in the walls or in the parenchyma of internal organs, in the vessels that supply the viscera, or in the serosal membranes that cover them (Loewy and Spyer, 1990). If the majority of these afferents transmit information from the viscera to the

central nervous system, there are some of them that also make contact with sympathetic preganglionic neurons in prevertebral ganglia (Matthews & Cuello, 1984). These anatomical relations indicate that in addition to their central actions visceral primary afferents may also play a role as part of peripheral regulatory reflexes (Matthews & Cuello, 1984) mainly those that are active in pathological conditions through positive feedback mechanisms. The same type of anatomical pattern is seen in enteric afferent fibres that, running in mesenteric nerves, can reach prevertebral sympathetic ganglia suggesting that enteric fibres also can participate in the regulation of visceral functions (Matthews & Cuello, 1984). The neuropeptides that appear to be involved in this sensory transmission are substance P, calcitonin gene-relate peptide and vasoactive intestinal peptide (Molander *et al.*, 1987; Sharkey *et al.*, 1987).

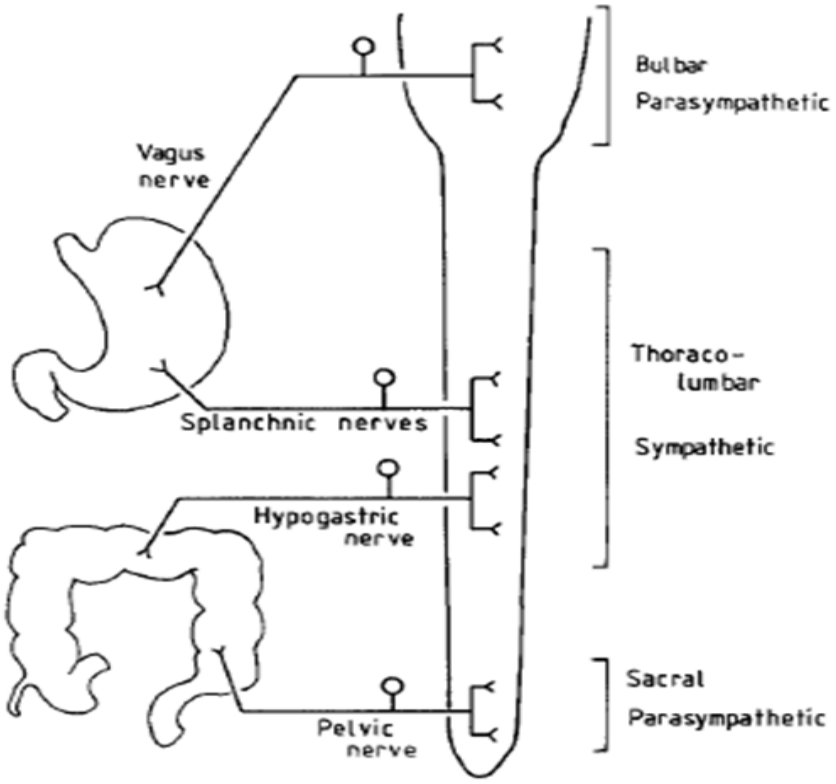


Figure 4-A. Drawing showing the dual afferent innervations of viscera according to their relative anatomical location in the body. From Loewy and Spyer, 1990.

The great majority of the viscera show dual afferent innervations with the larger majority of afferent fibres travelling in mixed parasympathetic nerves as the vagus and pelvic nerves (Andrews, 1986). There is not yet a conclusion about the physiological significance of this dual innervation – afferent fibres being carried in sympathetic and parasympathetic nerves- but data suggest that reflex and regulatory functions evoked by visceral stimulation are mainly triggered by activity in afferent fibres running in vagus and pelvic nerves while visceral sensation, and in particular visceral pain together with some visceral reflexes with origin in the mesenterium, is mediated by afferent fibres in sympathetic nerves (Loewy and Spyer, 1990, Jänig, 2008).

The efferent pathways

The efferent pathways of the autonomic reflex arc or the peripheral autonomic nervous system have three major subdivisions that are spatially segregated: the sympathetic, the parasympathetic, and the enteric nervous system. In other words, it can be said that the sympathetic and parasympathetic nervous systems consist in several functionally distinct subsystems each of them associated with a distinct type of target tissue (Jänig, 2008). They constitute the final autonomic pathway (Jänig & McLachlan, 1986) as each of them is based on a set of pre and postganglionic neurons that are synaptically connected in autonomic ganglia, constituting the connection between the brain centres and the target organ. These sets of neuronal pathways are the building blocks of the motor part of the autonomic reflex arc (Jänig, 2008).

It is generally accepted that, except for the enteric nervous system, the parasympathetic nervous pathways organization is simpler than the sympathetic one. If this can be true for some pathways and target organs like the pupillae and ciliary muscle seems unlikely to other target organs like the heart or the urinary bladder (Furness & Costa, 1987; Furness *et al.*, 2003; Furness, 2006; Jänig, 2008).

Each autonomic nerve pathway extending from the central nervous system (CNS) to an innervated organ is a two-neuron chain (Fig. 5-A) (except to the adrenal medulla that itself behaves as a sympathetic ganglion). The first neuron cell body, located in the CNS,

synapses with a second order neuron which cell body lies within an autonomic ganglion (Sherwood, 2010).

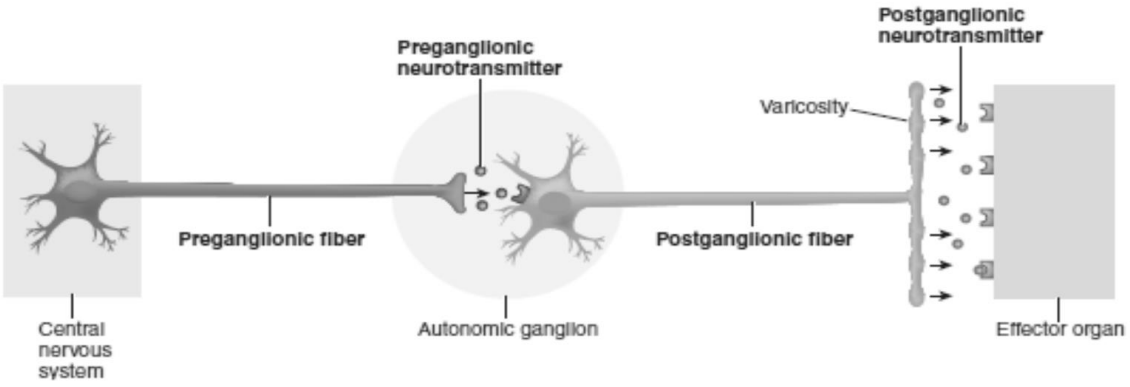


Figure 5-A. Schematic diagram of autonomic nerve pathway. From *Human Physiology: from Cells to Systems*, Sherwood, 2010.

The nerve fibres of the sympathetic and parasympathetic components are not present at all levels of brain-spinal cord axis. In fact, they leave the CNS at different levels - the sympathetic fibres from the thoracic and lumbar regions of the spinal cord, and the parasympathetic fibres from the brain and the sacral portion of the spinal cord. Therefore, the sympathetic division is also called the thoracolumbar division, whereas the parasympathetic is refered as the craniosacral division (Vander *et al.*, 2001).

I a. Sympathetic Nervous System

Sympathetic preganglionic neurons are an heterogeous population of neurons. Morphologically, they vary in somal shape, size and dendritic arborisation (Fig. 6-A) giving rise to either non-myelinated or myelinated axons, which are not singularly selective in relation to their target.

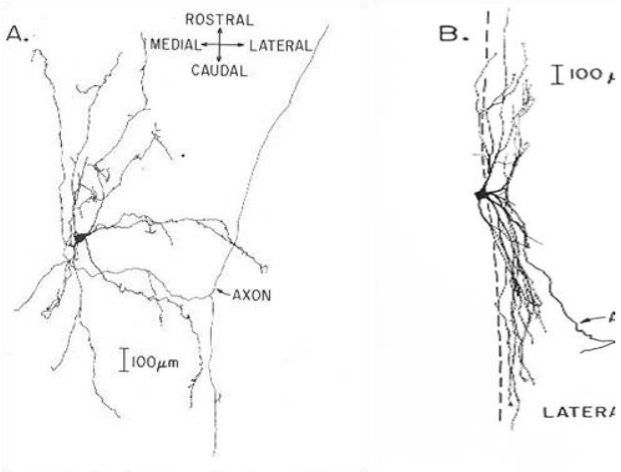


Figure 6-A. The sympathetic preganglionic neurons show several types of morphogical characteristics. Extracted from Cabot, 1990.

At the spinal cord, sympathetic preganglionic neurons are located in four nuclei: lateral funicular, intermediolateral, intercalated, and central autonomic nucleus of the spinal cord, being the most relevant for cardiovascular regulation the intermediolateral one (Fig. 7-A).

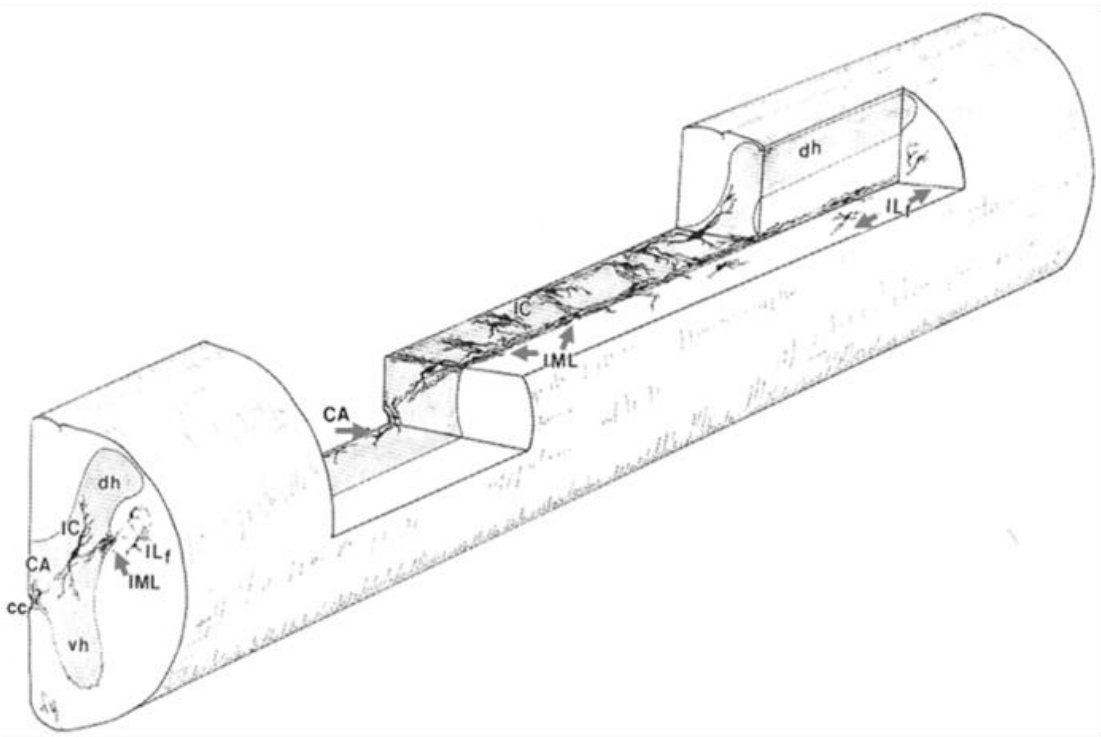


Figure 7-A. The sympathetic preganglionic cell bodies and axons show a characteristic "ladder" arrangement at the spinal cord. ILf, lateral funicular nucleus; IML, intermediolateral nucleus; IC,

intercalated nucleus; CA, central autonomic nucleus; dh, dorsal horn; vh, ventral horn; cc, central canal. Extracted from Cabot, 1990.

Independently of the way how preganglionic sympathetic neurons are posiotioned within the different spinal nuclei, they are segmentally organized providing, in this way, the anatomical substrate for a more general rostrocaudal functional topography (Fig 8-A) (Loewy and Spyer, 1990). Sympathetic preganglionic neurons leave the spinal cord only between the first thoracic and third lumbar segments, whereas sympathetic trunks extend the entire length of the cord, from the cervical levels high in the neck down to the sacral levels (Fig. 8-A)(Vander *et al.*, 2001).

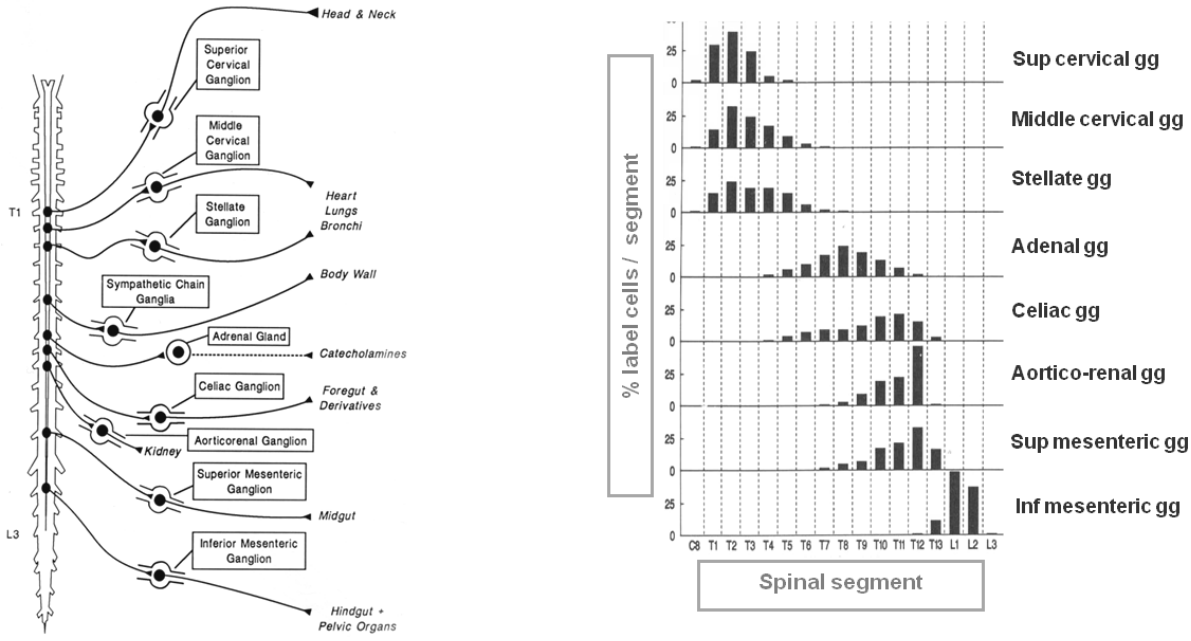


Figure 8-A. The segmental distribution of sympathetic preganglionic neurons (right) which reveals that most peripheral sympathetic ganglia receive dominant input from a single thoracic or lumbar spinal cord segment whereas those more caudally located receive sympathetic innervations from neurons located more caudally in the spinal cord (left). Extracted from Strack et al., 1988.

The sympathetic preganglionic neurons exhibit a low level of tonic activity from less than 1Hz up to 4Hz and their maximal frequency of discharge rarely exceeds the 20Hz. This may reflect the influence of both intrinsic membrane properties as well as the integration of excitatory and inhibitory potsynaptic potentials. A typical feature is a clear slowing of the depolarization rate due to calcium inward influx followed by an

afterhyperpolarization that involves an early phase mediated by potassium voltage dependent channels and a late phase dependent on calcium activated potassium channels. Preganglionic sympathetic neurons activity is regulated by segmental inputs from visceral and somatic afferents and supraspinal pathways through glutamatergic synapses and via N-methyl-D-aspartate (NMDA) and non-NMDA receptors which are inhibited by GABA receptors (Benarroch, 2006). According to their biophysical properties and their correlation with content of peptides and function, the sympathetic postganglionicneurons can be divided into three groups: phasic neurons (rapidly adapting); tonic neurons (slowly adapting) and neurons with a long after depolarization following an action potential (LAH neurons) (Adams and Harper, 1995, Tomikasa and Akasu, 1995, Jänig 2008). Almost all postganglionic neurons and some 15-25% of the preganglionic neurons are phasic neurons and, in addition, to norepinephrine they also have NPY as a co-transmitter. The tonic postganglionic neurons are numerous at prevertebral ganglia whereas the LAH neurons are also found in prevertebral ganglia and are almost exclusively located at celiac and superior mesenteric ganglia (Jänig, 2008).

I b. Parasympathetic Nervous System

Comparing with the amount of research on the sympathetic reflex responses, the studies on parasympathetic system are relatively few. The reasons are various but they all lie on the fact that the majority of the parasympathetic ganglia are located close or within the target wall organs and, thus, defining a very short postganglionic parasympathetic neuron. These less defined morphologically neuronal structures together with less exuberant parasympathetic innervation of the target organs when compared with the sympathetic one, lead to difficult activity recordings as well as to deficient peripheral modulation, either electrical or pharmacological, of these neuronal parasympathetic circuits. Also, evidences showing that some parasympathetic ganglia behave as simple relay stations also makes difficult to deeply understand parasympathetic function despite this lack of ganglionar functional integration is not so evident for cardiac and pelvic ganglia (Jänig, 2008; Keast, 1995, 1999).

In neuroanatomical terms, the preganglionic parasympathetic nuclei in the brain stem include the Edinger-Westphal nucleus (associated with cranial nerve III), the superior and inferior salivary nuclei (associated with cranial nerves VII and IX, respectively), and the dorsal motor vagal nucleus (DMNV) and the nucleus ambiguus (both associated with cranial nerve X). Preganglionic axons exit the brain stem through cranial nerves III, VII, and IX and project to postganglionic neurons in the ciliary, pterygopalatine, submandibular, and otic ganglia. Parasympathetic preganglionic fibers from the dorsal vagal nucleus project via the nerve X to postganglionic neurons embedded in thoracic and abdominal targets - the stomach, liver, gall bladder, pancreas, and upper intestinal tract (Fig. 9-A). Neurons of the ventrolateral nucleus ambiguus provide the main parasympathetic innervation of the cardiac ganglia, which innervate the heart, esophagus, and respiratory airways (Fig. 9-A; Kandel *et al.*, 2000).

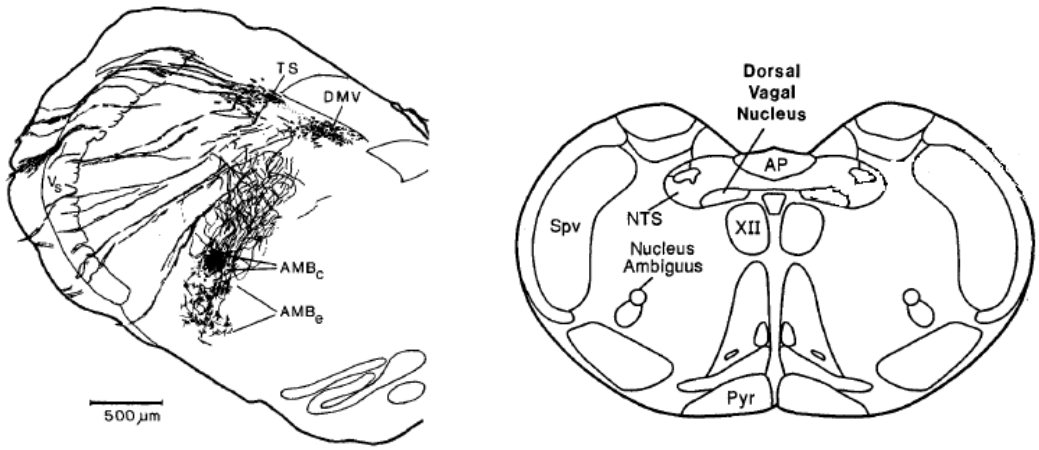


Figure 9-A. Transverse representation of vagal motor neurons from the nucleus ambiguous and dorsal motor nucleus of the vagus. On left, data from retrograde cell body labelling also shows the compact part—AMBC and the ventrolateral or external division—AMBe of nucleus ambiguus. TS tractus solitarius, DMV dorsal vagal nucleus, AP, area postrema, NTS nucleus tractus solitarius; Pyr pyramidal tract; Spv superior paraventricular nucleus (from Loewy and Spyer, 1990 and Bieger and Hopkins, 1987).

The heart is innervated by, at least, two parasympathetic pathways of central origin. One, acting directly on sinus node and other pacemaker cells, is involved in heart beat regulation and atrial inotropism. These myelinated neurons emerge from the nucleus ambiguus (Fig. 10-A) located in the caudal portion of the medulla and are activated by the baroreceptors' stimulation. They can show spontaneous and rhythmic activity, being the

absence of activity coincidental with inspiration whereas the activation is simultaneous to expiration. This coupling to the central respiratory activity is the basis of heart rate respiratory sinus arrhythmia. The second pathway is formed mainly by unmyelinated neurons that originate at dorsal motor nucleus of the vagus. Some of these neurons can also show spontaneous activity not modulated by the central respiratory drive or by baroreceptor activity and, upon the heart, their main function seems to generate coronary vasodilation when they are activated (Jänig, 2008, Izzo *et al.*, 1993; Feigl, 1998; Jones *et al.*, 1998; Cheng *et al.*, 1999).

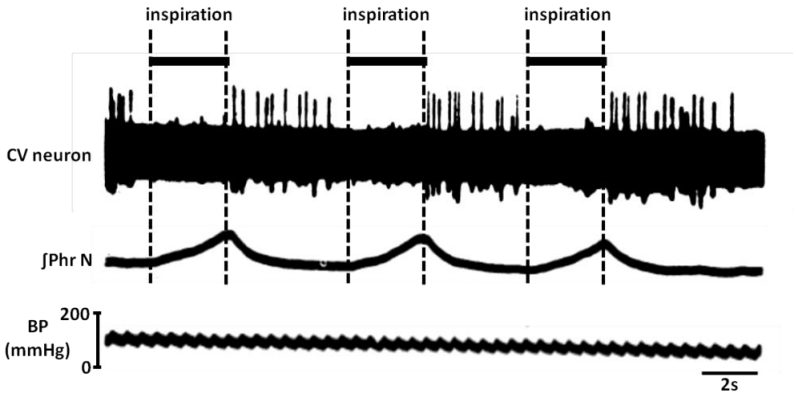


Figure 10-A- Discharge of parasympathetic cardiovascular neuron showing the cardiovascular-respiratory coupling. CV, cardiovascular; BP, blood pressure, ∫PhrN, integral of phrenic nerve activity.

The airways seem to be innervated by three parasympathetic pathways, two of them supplying the smooth muscle and the third one related to bronchial secretomotor neurons (Mazzone *et al.*, 2005) originating mainly at nucleus ambiguus (McAllen and Spyer, 1997). In this way, the first two pathways are responsible, respectively, for the cholinergic smooth muscle contraction under the activation of airway nociceptors, arterial chemoreceptors, upper airway and esophageal mechanoreceptors and the nitrergic relaxation of the smooth muscle related to the stimulation of nociceptors or rapidly adapting stretch afferents. Most of these neurons seem to be spontaneously active, being one type of them active during inspiration and postinspiration while, the other, is excited during expiration and hyperinflation. The broncho-secretomotor neurons seem to be activated by both pulmonary stretch receptors and nociceptors (Jänig, 2008,

Mitchell *et al.,* 1987, Jordan *et al*., 1997) but their activation type is unknown (Kesler *et al*., 2002).

The parasympathetic pathways to the gastrointestinal tract are rather complex with the preganglionic neurons emerging at the DMNV. These neurons are functionally differentiated to regulate the different gastrointestinal functions (Robertson *et al.*, 2012). There are also parasympathetic neurons innervating the salivary, and possibly the nasopharyngeal glands, which are located at the superior and inferior salivary nucleus. These neurons are of two types, motor (vasodilator) and secretor being mainly activated by chemical and mechanical stimuli at the naso-oro-pharyngeal cavity (Kim *et al.*, 2004; Bradley *et al.*, 2005; Fukami & Bradley, 2005).

In the eye, the pupillomotor and the vasodilator neurons are activated or inhibited by light while the accommodation pathway are specifically activated through target tracking (Jänig, 2008). These preganglionic neurons seems to be functionally organized at Edinger-Westphal nucleus with the pupilloconstrictor ones located more caudally whereas those involved in accommodation are located more lateral by opposition to the vasodilator of the choroidal circulation which have a medial predominance (Gamlin & Yoon, 2000).

In the sacral spinal cord, located at the second, third, and fourth sacral segments (S2-S4), the parasympathetic preganglionic neurons occupy the intermediolateral column. Axons of spinal parasympathetic neurons leave the spinal cord through the ventral roots between the caudal lumbar and sacral segments, and project in the pelvic nerve to the pelvic ganglion plexus (Robertson *et al.*, 2012). Pelvic ganglion neurons innervate the descending colon, bladder, and external genitalia (Fig. 11-A; (Kandel *et al.*, 2000). The sacral parasympathetic pathways to the hindgut are rather complex and little is understood in their physiology. They innervate the myenteric and serosal plexus, the last ones in a similar way of the sympathetic vasoconstrictor pathways to the gastrointestinal tract (Jänig, 2008). Apparently the sacral parasympathetic innervation is only involved in the control of gastrointestinal motility and in the control of defecation and continence. The lower urinary tract is supplied by, at least, two sacral parasympathetic pathways, one innervating the bladder body and, the other, the urethra. The first one leads to urinary bladder contraction whereas the last one, when activated, relaxes the urethra probably by the relase of NO and VIP (Michaelis *et al.*, 1996; Birder *et al.*, 2002; de Groat, 2002).

The sacral innervation of reproductive organs is also complex due to, not only, the type of target cells but, also, to the integration of lumbar sympathetic spinal systems and sacral spinal systems (McKenna, 1998, 1999, 2002). The spinal parasympathetic innervations of reproductive organs consists of, at least, one pathway innervating the erectile tissue in the man but the female innervation is not yet fully understood (McKenna, 1998, 1999; Giuliano *et al.*, 2001; McKenna, 2002).

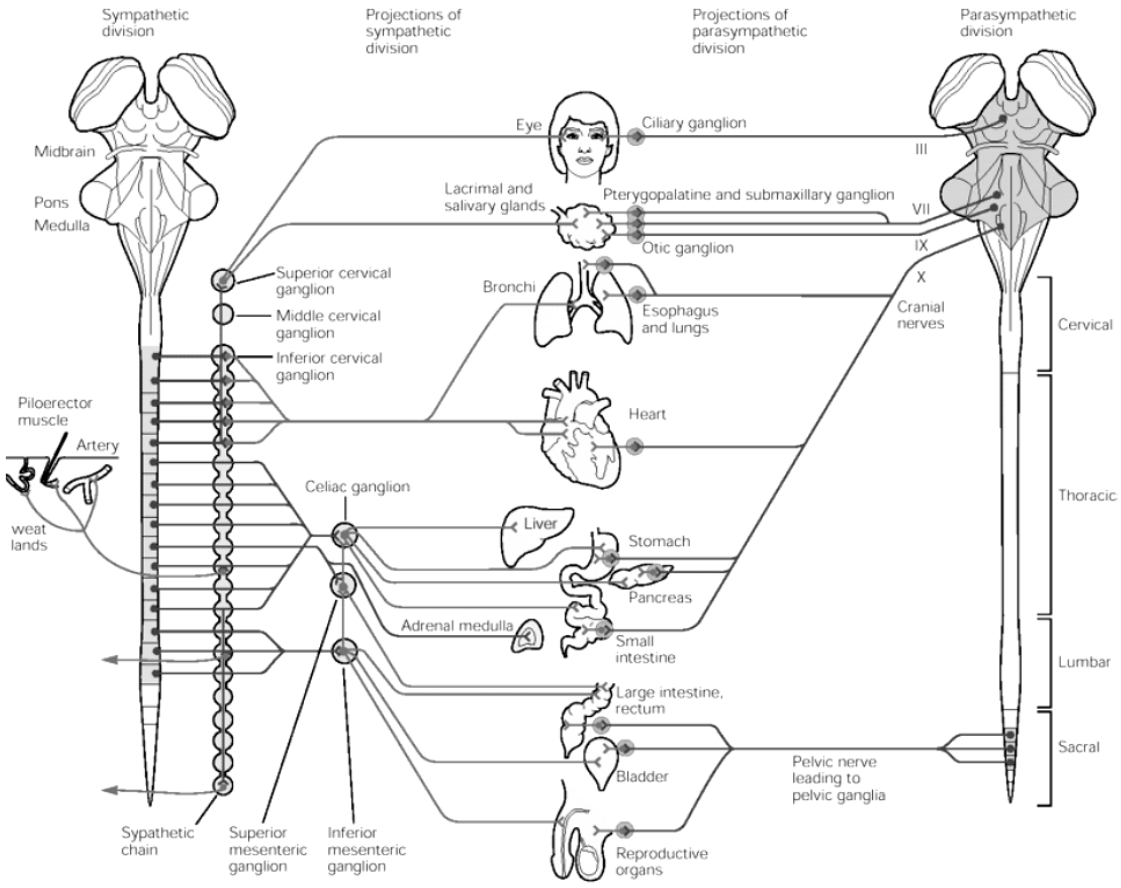


Figure 11-A. Sympathetic and parasympathetic divisions of the autonomic nervous system. Sympathetic preganglionic neurons are clustered in ganglia in the sympathetic chain alongside the spinal cord, extending from the first thoracic spinal segment to upper lumbar segments. Parasympathetic preganglionic neurons are located within the brain stem and in segments S2-S4 of the spinal cord. The major targets of autonomic control are shown here. Adapted from *Principles of Neural Science*, Kandel, 2000.

I c. Autonomic ganglia

The preganglionic efferent innervations to sympathetic neurons reside in ganglia dispersed in three arrangements: paravertebral, prevertebral, and previsceral or terminal ganglia. Paravertebral ganglia are paired structures that are located bilaterally along the vertebral column. They extend from the superior cervical ganglia, located rostrally at the bifurcation of the internal carotid arteries, to ganglia located in the sacral region (Fig. 8-A). There are three cervical ganglia (superior, middle and inferior cervical ganglion, which is usually termed the cervicothoracic or stellate ganglion), eleven thoracic ganglia, four lumbar ganglia, and four to five sacral ganglia. More caudally, two paravertebral ganglia join to become the ganglion impar. Prevertebral ganglia are midline structures located anterior to the aorta and vertebral column, and are represented by the celiac ganglia, aortic-renal ganglia, and the superior and inferior mesenteric ganglia. Previsceral or terminal ganglia are small collections of sympathetic ganglia located close to target structures (also referred to as short noradrenergic neurons). Generally, the sympathetic preganglionic fibers are relatively short and the postganglionic fibers are quite long in the SNS (Robertson *et al.*, 2012).

The target organs of sympathetic neurons include smooth muscle and cardiac muscle, glandular structures, and parenchymal organs, as well as, other cutaneous structures (Fig. 2-A; (Robertson *et al.*, 2012).

Some neurons of the cervical and upper thoracic ganglia innervate cranial blood vessels, sweat glands, and hair follicles; others innervate the glands and visceral organs, including the lacrimal and salivary glands, heart, lung and blood vessels. Neurons in the lower thoracic and lumbar paravertebral ganglia innervate peripheral blood vessels, sweat glands, and pilomotor smooth muscle (Fig. 11-A; Kandel *et al.*, 2000). Some preganglionic fibers pass through the sympathetic ganglia and branches of the splanchnic nerves to synapse on neurons of the prevertebral ganglia, which include the coeliac ganglion and the superior and inferior mesenteric ganglia (Fig. 11-A). Neurons in these ganglia innervate the gastrointestinal system and the accessory gastrointestinal organs, including the pancreas and liver, and also provide sympathetic innervation of the kidneys, bladder, and genitalia (Fig. 11-A). Another group of preganglionic axons runs in the thoracic splanchnic nerve into the abdomen and innervates the adrenal medulla, which is an

endocrine gland, secreting both epinephrine and norepinephrine into circulation. The cells of the adrenal medulla are developmentally and functionally related to postganglionic sympathetic neurons (Kandel *et al.*, 2000).

Sympathetic preganglionic neurons are cholinergic cells but some of them express, at least, one additional chemical phenotype. In particular, subpopulations of sympathetic preganglionic neurons contain the transmitter substances with or without co-localisation with acetylcholine like substance P, adrenaline, GABA, serotonin neurotensin, somatostatin, enkephalin, luteinizing hormone- releasing hormone, adenosine and ATP (Loewy & Spyer, 1990a; Burnstock, 2007).

The function of the sympathetic and parasympathetic neurons is also to distribute their information to the periphery having an anatomical and functional interface, which is the autonomic ganglia. At this level, the information is converged or diverged in order to match the size and type of target organ avoiding to increase the number of pos-ganglionic neurons that would be required to innervate a larger target organ (Purves, 1988; Voyvodic, 1989). The sympathetic preganglionic neurons converging to one postganglionic neuron originate from several contiguous spinal segments but the synaptic input of the postganglionic neuron is dominated by the spinal segment where the paravertebral ganglion lies (see above section Ia and Fig. 8-A). When stimulated, preganglionic axons converging on a postganglionic neuron elicit excitatory postsynaptic potentials (EPSP's) which depend on the number of acetylcholine quanta released, the membrane properties of the posganglionic neuron and the geometry of the synapse (McLachlan, 1995; Jamieson *et al.*, 2003). These excitatory inputs can be called "strong" if they elicit an action potential with the fast sodium current activated before the calcium current, or "weak" when less acetylcholine is released and the synaptic contacts are not associated with voltage sensitive calcium channels. In this way, spontaneously active neurons receive one or two preganglionic "strong" synaptic inputs and several "weak" ones not being normally activated physiologically by summation of the weak inputs because the firing rates of individual convergent inputs are too low (Jänig, 2008, McLachlan *et al.*, 1997). The prevertebral sympathetic ganglia have several functions like mediation of peripheral reflexes and the integration of impulse activity from spinal cord and the periphery.

The parasympathetic ganglia are different from the sympathetic ones not only anatomically but also functionally. Most of the parasympathetic postganglionic neurons receive a small number of synaptic inputs of "strong" nature and the parasympathetic ganglia seem to have only one relay function (Jänig, 2008).

I d. Dual autonomic innervation

The two divisions of the autonomic nervous system rarely operate independently, and autonomic responses generally represent the regulated interplay of both divisions (Table 1-A). The heart, glands and smooth muscles are innervated by both sympathetic and parasympathetic fibers; that is, they receive dual innervation. Whatever effect one division has on the effector cells, the other division usually has the opposite effect (exceptions to this rule are indicated in Table 1-A).

Moreover, the two divisions are usually activated reciprocally; that is, as the activity of one division is increased, the activity of the other is decreased. Dual innervation by nerve fibers that cause opposite responses provides a very fine degree of control over the effector organ. The sympathetic system promotes responses that prepare the body for stenuous physical activity in emergency or stressful situations, such as a physical threat from the outside. Indeed, a sympathetic response is characterized by an increase of heart rate, blood pressure and blood flow to the skeletal muscles, heart, and brain, release of glucose by the liver and pupils dilatation.

Simultaneously, activity of the gastrointestinal tract and blood flow to the skin are decreased by inhibitory sympathetic effects (Vander *et al.*, 2001; Sherwood, 2010; Table 1-A). The parasympathetic system dominates in quiet, relaxed situations. Under such nonthreatening circumstances, the body can be concerned with its own "general housekeeping" activities, such as digestion (Sherwood, 2010).

Table 1-A. Some Effects of Autonomic Nervous System Activity. Adapted from *Neural Control Mechanisms*, Vander, 2001.

Effector Organ	Receptor Type	Sympathetic Effect	Parasympathetic Effect
Eyes			
Iris muscle	Alpha	Contracts radial muscle (widens pupil)	Contracts sphincter muscle (makes pupil smaller)
Ciliary muscle	Beta	Relaxes (filattens lens for far vision)	Contracts (allow lens to become more convex for near vision)
Heart			
SA node	Beta	Increases heart rate	Decreases heart rate
Atria	Beta	Increases contractility	Decreases contractility
AV node	Beta	Increases conduction velocity	Decreases conduction velocity
Ventricles	Beta	Increases contractility	Decreases contractitily slightly
Arterioles			
Coronary	Alpha	Constricts	–
	Beta	Dilates	
Skin	Alpha	Constricts	–
Skeletal muscle	Alpha	Constricts	–
	Beta	Dilates	
Abdominal viscera	Alpha	Constricts	
	Beta	Dilates	–
Salivary glands	Alpha	Constricts	Dilates
Veins	Alpha	Constricts	–
	Beta	Dilates	
Lungs			
Bronchial muscle	Beta	Relaxes	Contracts
Bronchial glands	Alpha	Inhibits secretion	Stimulates secretion
	Beta	Stimulates secretion	
Salivary glands	Alpha	Stimulates watery secretion	Stimulates watery secretion
	Beta	Stimulates enzyme secretion	
Stomach			
Motility, tone	Alpha and Beta	Decreases	Increases
Sphincters	Alpha	Contracts	Relaxes
Secretion		Inhibits (?)	Stimulates
Intestine			
Motility	Alpha and Beta	Decreases	Increases
Sphincters	Alpha	Contracts (usually)	Relaxes (usually)
Secretion	Alpha	Inhibits	Stimulates
Gallbladder	Beta	Relaxes	Contracts
Liver	Alpha and Beta	Glycogenolysis and gluconeogenesis	–
Pancreas			
Exocrine glands	Alpha	Inhibits secretion	Stimulates secretion
Endocrine glands	Alpha	Inhibits secretion	–
	Beta	Stimulates secretion	
Fat cells	Alpha and Beta	Increases fat breakdown	–
Kidneys	Beta	Increases rennin secretion	–
Urinary bladder			
Bladder wall	Beta	Relaxes	Contracts
Sphincter	Alpha	Contracts	Relaxes
Uterus	Alpha	Contracts in pregnancy	Variable
	Beta	Relaxes	
Reproductive tract (male)	Alpha	Ejaculation	Erection
Skin			
Muscles causing hair erection	Alpha	Contracts	–
Sweat glands	Alpha	Localized secretion	Generalized secretion
Lacrimal glands	Alpha	Secretion	Secretion

There are several exceptions to the general rule of dual reciprocal innervation by the two branches of the autonomic nervous system. The innervated blood vessels (most arterioles and veins are innervated; arteries and capillaries are not) receive only sympathetic nerve

fibers. Regulation is accomplished by increasing or decreasing the firing rate above or below the tonic level in these sympathetic fibers. The only blood vessels to receive both sympathetic and parasympathetic fibers are those supplying the penis and clitoris. Most sweat glands are innervated only by sympathetic nerves. The postganglionic fibers of these nerves are unusual because they secrete acetylcholine rather than norepinephrine. Salivary glands are innervated by both autonomic divisions, but sympathetic and parasympathetic activity is not antagonistic. Both stimulate salivary secretion, but the saliva's volume and composition differ, depending on which autonomic branch is dominant (Sherwood, 2010).

I e. Autonomic Neurotransmission

The transmission principles at the autonomic nervous system were originally defined on the release or acetylcholine or norepinephrine. However, the current knowledge indicates that together with these two classical neurotransmitters, other co-transmitters as well as neuropeptides are involved in autonomic transmission at several levels.

The sympathetic and parasympathetic preganglionic fibers release the same neurotransmitter, acetylcholine (ACh), but the postganglionic endings of these two systems show different types of neurotransmission. Most of the postganglionic sympathetic neurons release norepinephrine (NE) but the sympathetic sudomotor and the muscle vasodilators transmission is cholinergic in nature as well as in the parasympathetic postganglionic neurons. Epinephrine is released by the adrenal medulla. These cathecolamines and their precursor dopamine are synthesized from L-tyrosine by the action of tyrosine hydroxylase (Fig. 12-A) (Benarroch, 2006).

Acetylcholine (Ach) is the neurotransmitter of preganglionic sympathetic and parasympathetic neurons as well as parasympathetic ganglion cells and sympathetic neurons innervating the sweat glands. ACh exerts its effects through two classes of receptors, nicotinic and muscarinic, the first mediating the fast transmission between the preganglionic fibres to the autonomic ganglia while muscarinic receptors are responsible for the effects of Ach on target organs and modulate the excitability of autonomic ganglion neurons and pre-synaptic neurons (Benarroch, 2006).

Two main categories of receptors -M1 and M2 types – enclose five types of muscarinic receptors. The M1 type receptors include the M1-, M3- and M5- receptors (Benarroch, 2006). M1 stimulate gastric secretion in the stomach whereas M3 mediate excitatory effects of Ach on smooth muscle and exocrine secretion. The M2-type (M2 and M4) mediate the pre and postsynaptic inhibitory effects of ACh (Benarroch, 2006) (Fig. 13-A).

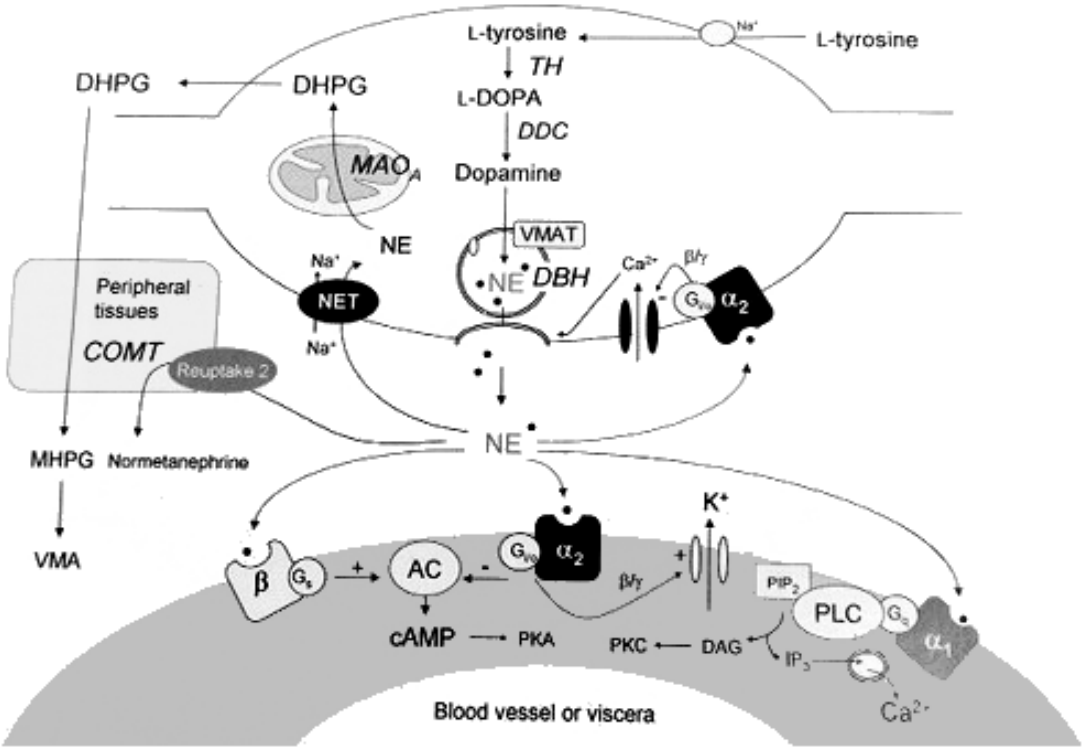


Figure 12-A. Diagram showing the biochemical pathways of catecholamine release at synaptic terminals. NE release is primarily inhibited by α2- autoreceptors and together with epinephrine impress α1, α1 and β-adrenoreceptors. AC, adenylate cyclase; Ach, acetylcholine; COMT, catechol O-metyltransferase; DAG, diacylglycerol; DBH, dopamine β-hydroxylase; DDC, Dopa decarboxylase;DHPG, dihydroxyphenylglycol; IP_3, inositol triphosphate; MAO_A, monoamine oxidase A; MHPG, methoxyhydroxyphenylglycol; NE, norepinephrine; NET, norepinephrine transporter; PIP_2, phosphatidylinositol biphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; TH, Tyrosine hydroxylase; VMA, vanillylmandelic acid; VMAT, H+/adenosine triphosphate-dependent vesicular monoamine transporter; Extracted from Benarroch, 2006.

However, for autonomic postganglionic neurons, muscarinic and cathecolaminergic blockers do not abolish completely their activation, suggesting that autonomic excitation is also dependent of other forms of neurotransmission. That is the case with the NANC

(NorAdrenergic NorCholinergic) signalling which is also observed at autonomic synapses. The NANC transmission is particularly important in the enteric nervous system and involves nitric oxide (NO) and purines, in particularly adenosine triphosphate (ATP) acting on P2 receptors and adenosine acting via P1 receptors. The autonomic classical neuro- and co-transmission is complemented with the release of neuropeptides like neuropeptide Y (NPY), vasoactive intestinal peptide (VIP) and galanin (GAL). There are also other peptides can be effectively involved at autonomic synapses like tachykinins, calcitocin gene related peptide (CGRP) but their interaction

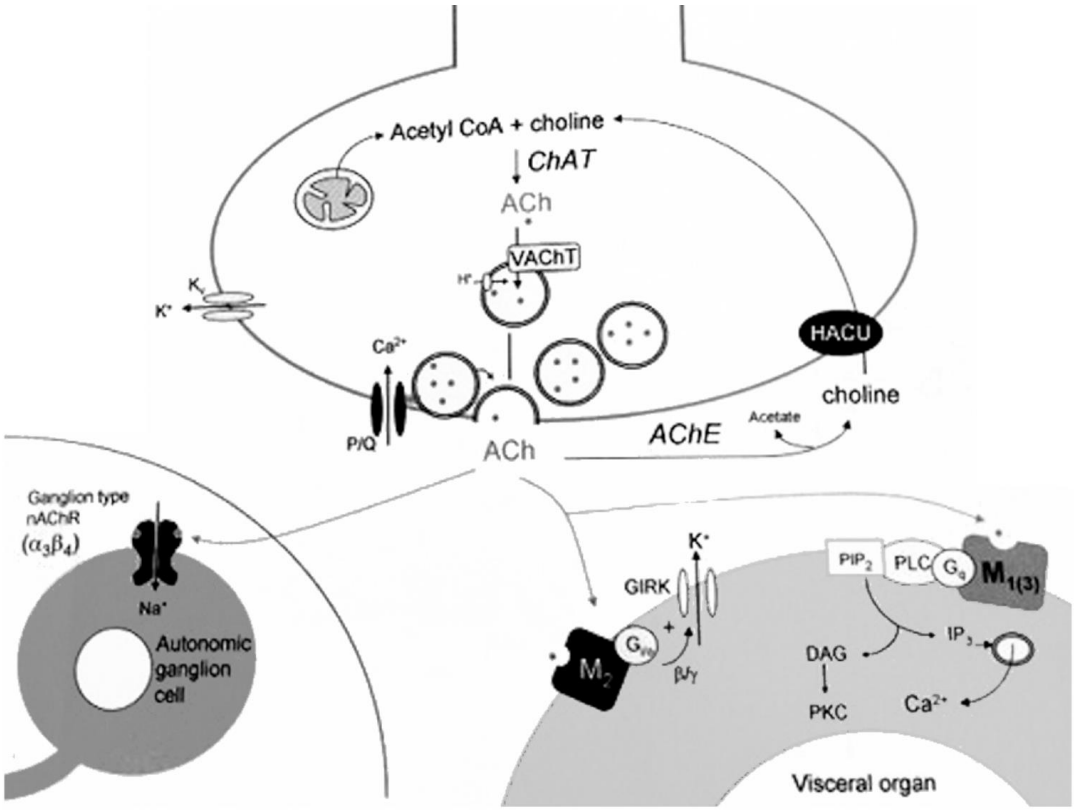


Figure 13-A. Mechanisms of ACh synthesis, storage, release and metabolism. Acetyl CoA, acetyl coenzyme A; Ach, acetylcholine; AChE, acetylcholinesterase; ChAT, choline acetyltransferase; DAG, diacylglycerol; GIRK, inward-rectifying K+ channels; HACU, high-affinity choline uptake; IP_3, inositol triphosphate; nAChR, nicotinic ACh receptors; PIP_2, phosphatidylinositol biphosphate; PKC, protein kinase C; PLC, phospholipase C; VAChT, vesicular ACh transporter; Extracted from Benarroch , 2006.

within autonomic neurotransmission processes is still a matter of debate. NPY is mainly associated to postganglionic sympathetic vasoconstrictors neurons acting complementary with norepinephrine. However, in the heart, it attenuates the decrease of heart rate

elicited by the parasympathetic activation (Potter, 1987). In opposition, VIP seems to have mainly vasodilator actions reinforcing acetylcholine dilator actions (Gibbins *et al.*, 1984; Lundberg, 1996). Apparently, vasoconstriction in some vascular beds, like the skin and mesentery, could have also the interference of substance P and CGRP, being vasodilation the final effect (Holzer, 1992; Häbler *et al.*, 1997; Häbler *et al.*, 1999).

I f. Central autonomic network

Central autonomic pathways are organized at two levels of complexity. Some pathways are organized for reflex adjustments of the end organ and others are organized in a more complex way by connecting to higher neural centers that form a central autonomic circuit capable of producing widespread autonomic, endocrine, and behavioral responses (Loewvy and Spyer, 1990) through preganglionic sympathetic and parasympathetic neurons, pituitary and peripheral endocrine organs and motoneurons innervating the respiratory and sphincteric muscles, respectively (Benarroch, 2006).

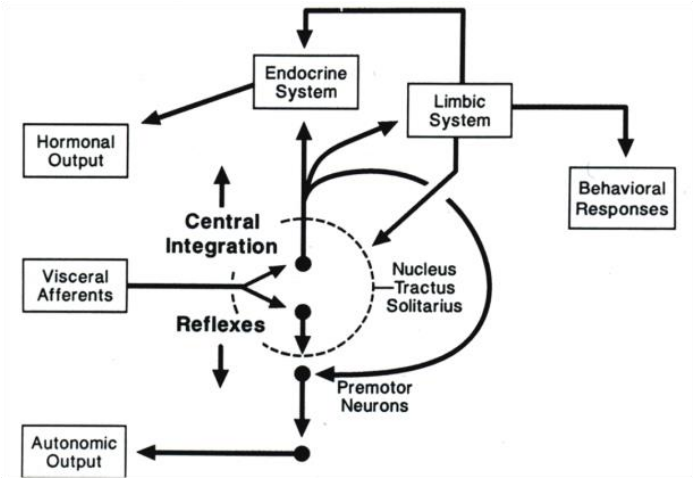


Figure 14-A. Drawing depicting the two main types of visceral information processing by the central autonomic network. Information with origin in periphery is processed either for reflex responses or for an integrated autonomic, hormonal and behavioral output which prototype is thermoregulation at hypothalamic level. Extracted from Loewy and Spyer, 1990.

Central control of autonomic function involves several interconnected areas distributed throughout the neuraxis (Robertson *et al.*, 2012). This central autonomic network has a critical role in moment-to-moment control of visceral function, homeostasis, and adaptation to internal or external challenges. In particular, it receives and integrates

information with origin in several sources: a) sensory information with visceral, nociceptive, thermal and muscular origin; b) limbic information through the central nucleus of the amygdala; c) humoral inputs directly or via circumventricular organs; d) information with origin in central oscillators that regulate the pacemaker cells at suprachiasmatic nucleus; and, e) information with origin in the pathways regulating the sleep-wake cycle (Loewy and Spyer, 1990, Benarroch, 2006, Rocha, 2009, (Robertson *et al.*, 2012, Mathias & Bannister, 2012).

The functions of the central autonomic network are organized in four hierarchical levels that are closely interconnected: spinal, bulbopontine, pontomesencephalic and forebrain levels (Fig. 15-A).

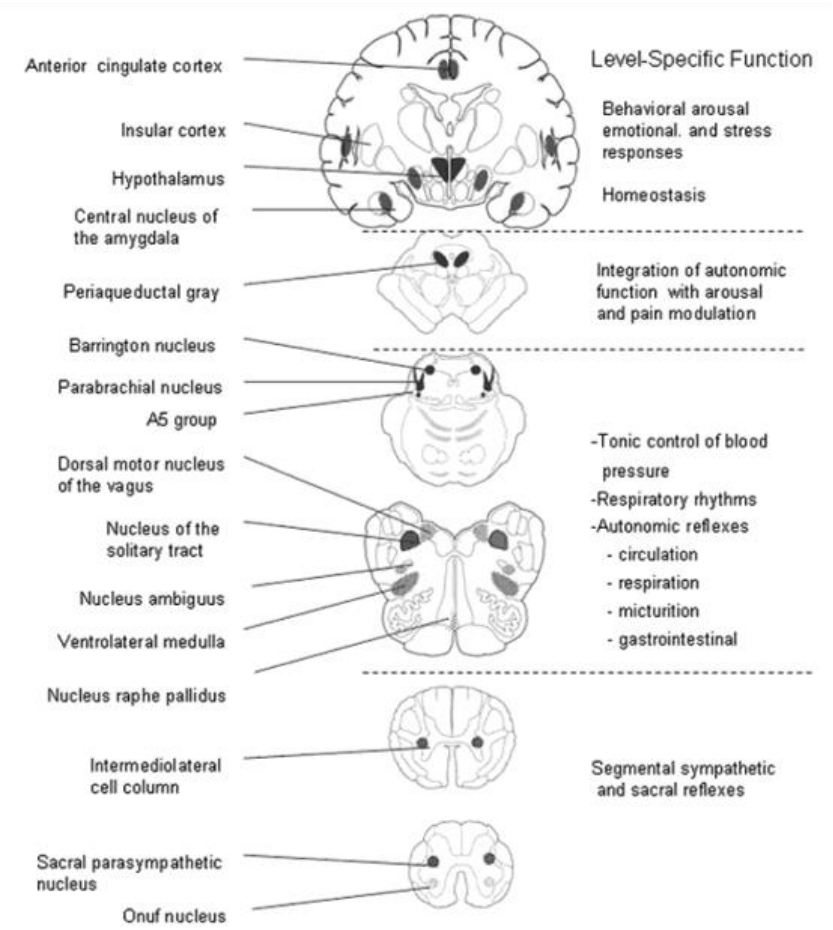


Figure 15-A. Central autonomic control areas and levels of interaction of autonomic control. Extracted from Robertson, 2012.

These areas are reciprocally interconnected. In fact, they receive convergent inputs of visceral and somatic nature and generate stimulus specific profiles of autonomic, endocrine and motor responses which are regulated to the behavioural state. The spinal level mediates segmental sympathetic or sacral parasympathetic reflexes and is engaged in stimulus-specific patterned responses under the influence of the other levels.

The bulbopontine (lower brainstem) level is involved in reflex control of circulation, respiration, gastrointestinal function, and micturition. In particular, at this level, is located the nucleus of solitary tract, which is the primary relay station for the reception of peripheral visceral information, as well as, the ventrolateral medulla (RVLM), which contains bulbospinal neurons that are fundamental for the vasomotor, cardiac and respiratory functions control and for the coordination of several cardiovascular reflexes. These RVLM neurons also control hypothalamic function and neurons of ventral respiratory group involved in respiratory rhythmogenesis (Loewy and Spyer, 1990, Benarroch, 2006, Rocha, 2009, (Robertson *et al.*, 2012, Mathias & Bannister, 2012). Located more rostrally, the parabrachial nucleus is a major relay center for the convergence of sensory information of several natures: visceral, nociceptive and thermoreceptive and contains separate subnuclei linked to taste, salivation, gastrointestinal, cardiovascular and respiratory regulation together with clusters of neurons involved in osmo- and thermoregulation (Loewy and Spyer, 1990, Benarroch, 2006, Rocha, 2009, Robertson *et al.*, 2012, Mathias & Bannister, 2012).

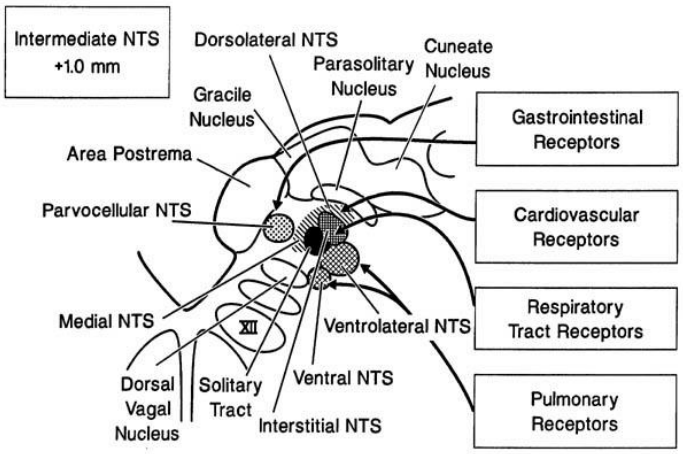


Figure 16-A. Drawing representing NTS visceral organization. In this way, sensory information originated from different visceral organs is conveyed to the same cluster of NTS cells. NTS also shows a functional

organization, where the convergence of sensory information is stimuli specific (Silva Carvalho et al, 1997) (Extracted from Loewy and Spyer, 1990)

The midbrain periaqueductal gray matter (PAG) is involved in the integration of autonomic, somatic and antinociceptive responses to stressful stimuli. Morphologically, PAG is divided into columns which control cardiorespiratory and urinary function as well as pain, thermoregulation and reproductive function including vocalization (Loewy and Spyer, 1990, Benarroch, 2006, Rocha, 2009, Robertson *et al.*, 2012, Mathias & Bannister, 2012).

The forebrain level includes the hypothalamus and components of the anterior limbic circuit, including the insular cortex, anterior cingulated cortex and amygdala. The hypothalamus which contains three main areas- periventricular linked to neuroendocrine control, lateral involved in arousal and a medial zone controlling behaviour- has a central role in neuroendocrine integration being critical for the homeostasis and integrative adaptive responses. The hypothalamus is also the location where the autonomic, endocrine and immune system communicate. Also regulates sleep –wake cycle, body temperature, food intake, osmolarity and fluid balance. Hypothalamic nuclei contain neurons that project to preganglionic neurons and in particular those cells of the paraventricular nucleus are involved in the stress response (Loewy and Spyer, 1990, Benarroch, 2006, Rocha, 2009, Robertson *et al.*, 2012, Mathias & Bannister, 2012).

The hypothalamus together with PAG are also involved in the defense reaction, an acute but active reaction of adaptation to stressful stimuli with lead to sympathetic activation with tachycardia, hypertension, positive inotropism, increase in stroke volume and cardiac output, redistribution of blood flow, tachypneia and inhibition of baroreflex and facilitation of the chemoreceptor reflex (Silva Carvalho et al, 1995a,b). In this situation, the paraventricular nucleus coordinates the neuroendocrine integration that includes sympathoexcitation and secretion of vasopressin and activation of the adrenomedullary and adrenocortical systems (Loewy and Spyer, 1990, Benarroch, 2006, Rocha, 2009, Robertson *et al.*, 2012, Mathias & Bannister, 2012).

The amygdala, in particular the central nucleus, initiates the endocrine, autonomic and motor outputs that are critical for the expression of emotions included conditioned

behaviours. The anterior cingulated cortex at the insular cortex, which is organized in a viscerotopic way, is responsible for the initiation of autonomic responses related to motivation and goal-directed behaviours (Jänig, 2008). The anterior cyngulate gyrus, ventromedial prefrontal cortex, amygdala, striatum, hypothalamus and PAG form a functional unit involved in the assessment of emotional content of stimuli and in the context-dependent autonomic, endocrine and motor outputs play a critical role in the integrated responses to stress, emotional responses and motivated behaviour.

II. Autonomic Nervous System Evaluation

II a. Autonomic manoeuvres and Ewing battery of tests

Autonomic dysfunction may result from primary modifications of the autonomic nervous system or secondary to a wide range of diseases eliciting severe morbidity and mortality. Together with a detailed history and physical examination, laboratory autonomic evaluation has became essential for the evaluation of several clinical conditions and the establishment of effective therapeutic schemes more personalized and refined. There are several standard autonomic provocative manoeuvres which goal is to test the system with a stimulus of supra-threshold to maximal intensity in order to observe the target organs evoked responses in terms of presence/absence, duration and magnitude. These manoeuvres should be performed at an autonomic laboratory which should fulfil several requirements: temperature and humidity control (20-23ºC and 25-35%, respectively) with an area of around 20 square metres. All tests should be performed by experienced technicians under medical supervision and, depending on the type of evaluation, their required number can be of two per patient. Technicians' training is critical to the successful performance of an autonomic test battery and cannot be stressed enough (Low & Benarroch, 2008; Mathias & Bannister, 2012). Technicians' must be familiar with sudometrics, ECG, beat to beat BP and blood flow recordings, they must have a practical understanding of computers and must be able to recognize technical problems and their management as well as be knowledgeable in electrical safety and recognition of the main ECG abnormalities and be trained in cardiopulmonary resuscitation (Low & Benarroch, 2008; Mathias & Bannister, 2012). There are some patient's preparation requirements

which should be followed. No food and tobacco is allowed, at least 4 hours, before the study, alcohol is not permitted, at least for 12 hours before the study and compressive clothing should not be used in the morning of the test which should, preferentially be performed in the morning period. Medications should be discontinued according with the drugs half-life and the patient condition and, particularly, those that affect directly the autonomic nervous system. Due to the large intra and inter-individual variability, normative data values are per laboratory and should be grouped by sex, age and decades of life. There are different ways of categorising autonomic tests which take into account the target system, the type of variables recorded and the degree of invasion. Usually, and due to the nature of the recording devices, most of the manoeuvres are targeting the cardiovascular system and are non-invasive in nature (see table 2-A).

Table 2-A. Summary of the autonomic provocative manoeuvres using for autonomic evaluation (Extracted fom Mathias and Bannister, 2012).

Cardiovascular	Head-up tilt (60°) Standing; Valsalva manoeuvre Pressure stimuli–isometric exercise, cutaneous cold, mental arithmetic Heart rate responses-deep breathing, hyperventilation standing, head-up tilt Liquid meal challenge Exercise testing Carotid sinus massage, head and neck movements 24-hour ambulatory blood pressure and heart rate monitoring
Biochemical	Plasma noradrenaline, adrenaline, dopamine–supine and head-up tilt or standing urinary catecholamines; plasma rennin activity and aldosterone
Pharmacological	Noradrenaline–α-adrenoceptors, vascular Isoprenaline–β-adrenoceptors, vascular and cardiac Tyramine–pressor and noradrenaline response Edrophonium–noradrenaline response Atropine–parasympathetic cardiac blockade Clonidine–stimulation or suppression test
Sudomotor	Central regulation–thermoregulatory sweat test Sweat gland response-intradermal acetylcholine, quantitative sudomotor axon reflex test (QSART), localized sweat test Sympathetic skin response, sympathetic vasomotor response
Gastrointestinal	Barium studies, videocinefluoroscopy, endoscopy, gastric emptying studies
Renal function and urinary tract	Day and night urine volumes and sodium/potassium excretion Urodynamic studies, intravenous urography, ultrasound examination, sphincter electromyography
Sexual function	Penile plethysmography Intracavernosal papaverine
Respiratory	Laryngoscopy Sleep studies to assess apnoea/oxygen desnaturation
Eye	Schirmer's test Pupillary function–pharmacological and physiological

The evaluation protocol as well as the data analysis protocol should be designed appropriately to the study. One standard and the most common evaluation protocol is called the Ewing battery (Ewing et al, 1985) which comprises the assessment of heart rate response to deep metronomic breathing, blood pressures changes upon isometric hand-grip and blood pressure and heart rate responses to Valsalva manoeuvre and active standing (Ducla-Soares *et al.*, 2007; Low & Benarroch, 2008; Xavier *et al.*, 2008; Mathias & Bannister, 2012, Lahrmann et al, 2011).

Additional non-invasive manoeuvres like cold pressure test, mental stress and tilt table are also currently used in autonomic evaluation.

The Valsalva manoeuvre assesses the sympathetic and the parasympathetic reaction to baroreflex activation when a subject maintains an expiratory pressure of 40mmHg for 15 seconds with an open glottis. The test responses are divided into four phases, two of which are reflex in nature (II and IV) and two mechanical (I and III). Results depend on the position, age and gender of the subject, as well as the duration and intensity of the expiratory pressure. In patients with autonomic dysfunction, typically there is a loss of both the BP overshoot and the reflex bradycardia (Lahrmann et al, 2011) (Fig. 17-A).

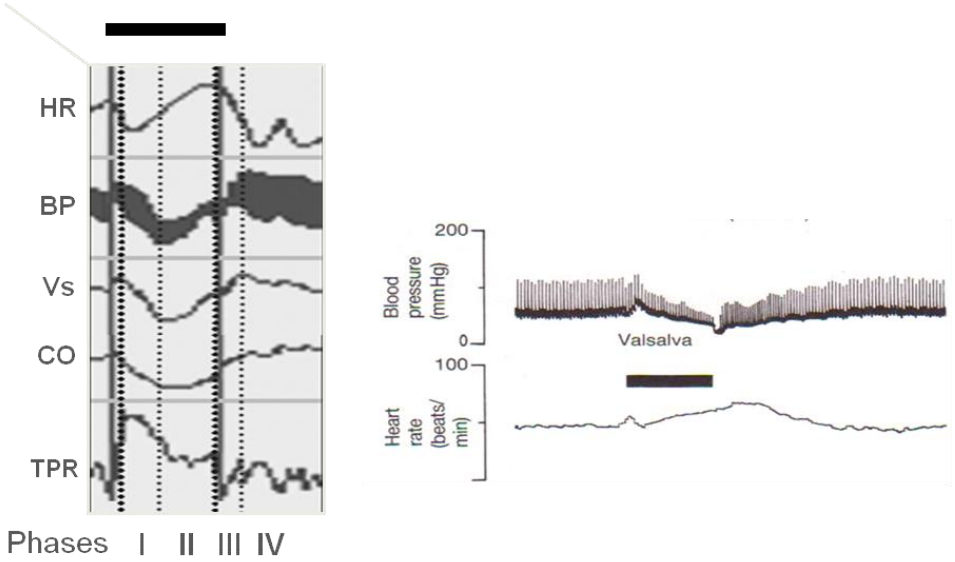


Figure 17-A. The Valsalva manoeuvre. On the left, data from a normal subject: following a brief decrease in HR and increase in BP due to aorta compression (I), BP first decreases and then increases (II); at III, due to the strain release, a consecutive decline in BP and increase in HR is observed which precedes a BP overshoot due to persistent sympathetic activity together with normalisation of venous return (IV). The increased BP mediates baroreflex-induced bradycardia and is quantified using the Valsalva ratio (i.e. the

ratio of the highest HR (II) vs the lowest HR (IV)). Results depend on the position, age and gender of the subject, as well as the duration and intensity of the expiratory pressure. (data extracted from CNSystems, Graz). On the right, a Valsalva manoeuvre in a patient with disautonomy showing the absence of phase IV (from Mathias and Bannister, 2008). In patients with autonomic dysfunction, typically there is a loss of both the BP overshoot and the reflex bradycardia Vs: stroke volume; Co: cardiac output; TPR: total peripheral resistance

On deep (metronomic) breathing HR autonomic function is assessed with the patient breathing metronomically at a rate of six cycles/minute for three minutes, when respiratory sinus arrhythmia is maximal. Changes of HR with deep respiration can be considered a parameter of parasympathetic cardiac control (Lahrmann et al, 2011; Low & Benarroch, 2008; Mathias & Bannister, 2012)).

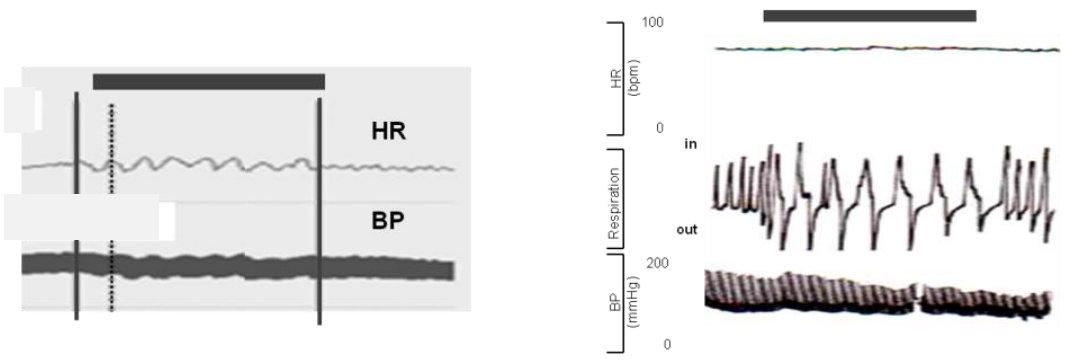


Figure 18-A. HR responses on deep breathing. On the left, a normal subject (from CNSystem, Graz) and on the right, data from a patient with disautonomy where the imprints of respiration are not observed in HR recording (from Mathias and Bannister, 2006)

HR variability during deep breathing is influenced by the position of the test subject, the rate and depth of breathing, hypocapnia, sympathetic activity, bodyweight and use of salicylates and other drugs (Larhmann et al, 2011).

To evaluate cardiovascular changes upon active orthostatic challenge (Fig. 19-A), Ewing introduced two concepts: the 30/15 ratio for HR and BP fall analysis which when corresponds to a fall in systolic pressure of at least 20mmHg or in diastolic pressure of at least 10mmHg within three minutes of standing or head-up tilt defines orthostatic hypotension (Lahrmann et al, 2011, Low and Sletten, 2009, Mathias and Bannister, 2008, 2013). The 30/15 ratio corresponds to the ratio between the shortest RR interval around

the 15th heart beat and the longest RR interval around the 30th heart beat after standing up.

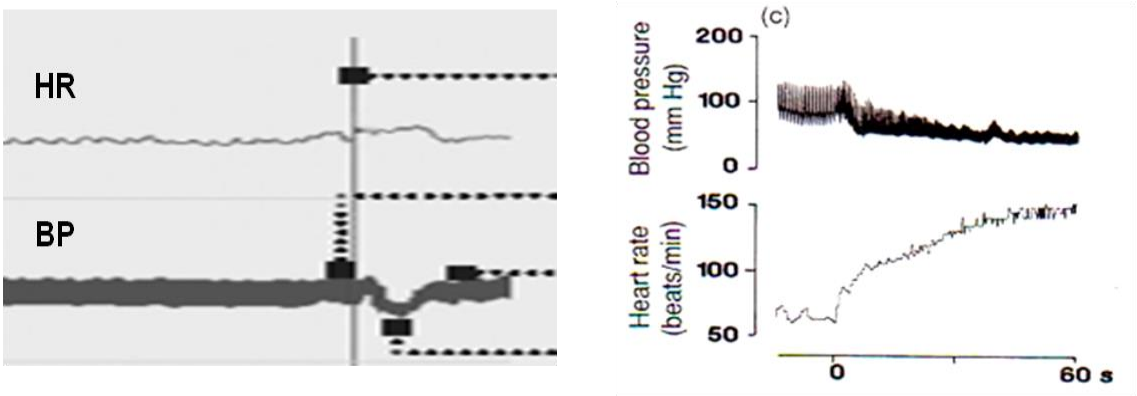


Figure 19-A. Active standing evaluates the simultaneous acute changes on BP and HR. On the left, a normal response (from CNSystems, Graz) and on the right, responses elicited by a patient with autonomic failure: in this case, HR increases strongly in an attempt to compensate baroreceptor impairment and the continuous decrease of BP (from Mathias and Bannister, 2006).

To complement the autonomic evaluation of active standing, the head up tilt test (HUT) is currently performed. Conceptually, it allows the detection of the haemodynamic modifications elicited by baroreceptor reflex activation without the interference of the muscular pump of the legs. However, rarely it occurs, in this conceptually way, as subjects usually develop an alert reaction at the initial stages of the bed tilting which superimposes in visual terms to the changes on BP and HR elicited by baroreflex activation. Classically, haemodynamic changes associated with HUT have two phases: an initial cardiovascular acute response with a duration of 30 seconds, and a stabilisation phase composed of two periods – an adaptation period occurring one to two minutes after orthostasis, and a later response to prolonged orthostasis lasting for more than five minutes (Fig 20-A) (Lahrmann *et al*, 2011, Low & Benarroch, 2008; Mathias & Bannister, 2012).

The cutaneous cold test evaluates sympathetic activation mediated by nociceptors, which is observed mainly through BP changes, upon hand until the arm immersion in a freezing cold water at 4ºC (Fig. 21-A).

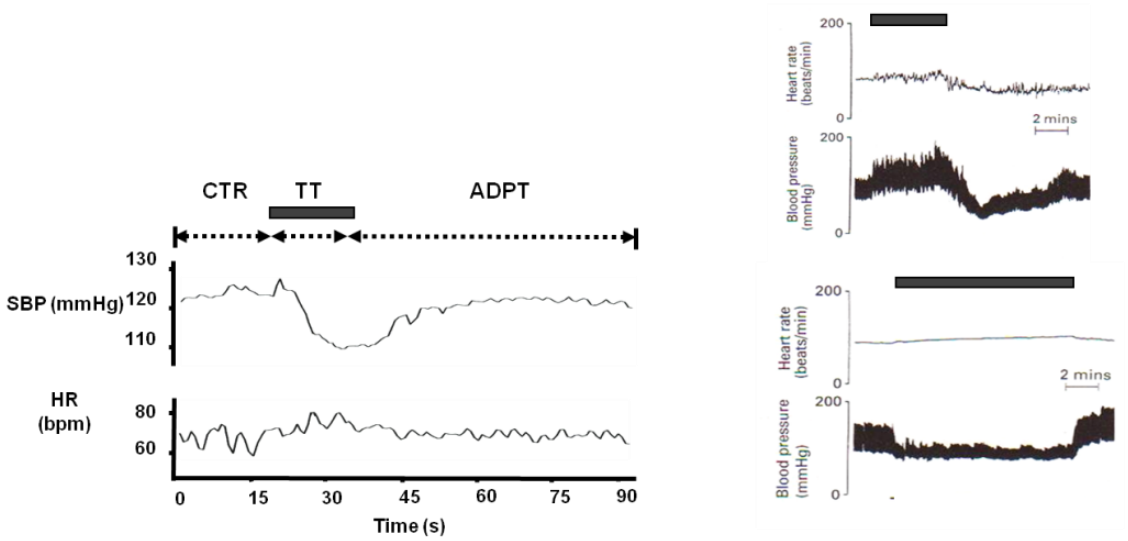


Figure 20-A. The normal HR and BP responses to HUT (left, Ducla Soares et al, 2007)**.** To perform this manouvre the subject is lying down in a tilt test table, being tilt up at 60-70º after a period of resting of, at least, 5minutes. On the right, two examples, of abnormal responses from two patients with different degrees of autonomic impairment: on top, a late adaptation is shown, whereas in the bottom the patient was unable to adapt and the bed had to return to the initial position before the end of the test (from Mathias and Bannister, 2006).

This test, which is a predominantly sympathetic test, differs from the cold face test by which the application of cold stimulus to the face, stimulates the trigeminal nerve and elicits bradycardia. On the background of the cold face test is the diving response. (For more and deep information on autonomic evaluation tests see Mathias and Bannister 2012 and Low and Benarroch, 2008).

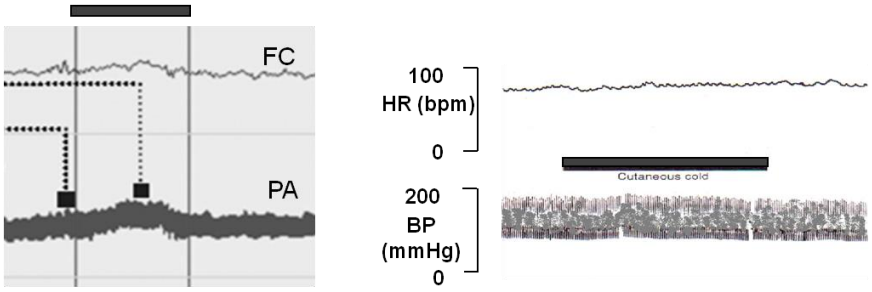


Figure 21-A. The cutaneous cold test is likewise the hand grip and the mental stress, a test that evaluates mainly adrenergic function. All of them, have the physiological feature of integrating at cortical level. On the left, a normal response (CNSystems, Graz) and on the right a response of a patient with disautonomy

showing the absence of sympathetic activation, well observed in the absence of BP changes (Mathias and Bannister, 2008).

II b. Sudomotor function

Sudomotor function complements the cardiovascular autonomic testing as impairment of sweating, either by hypo- or hyperhidrosis, focal or generalised, are quite frequently associate to autonomic failure. There are several tests available categorized according to its qualitative, semi-quantitative or quantitative nature and ability to evaluate central or peripheral sudomotor function. The thermoregulatory sweat test (TST), the quantitative sudomotor axon reflex test (QSART), the sympathetic skin response test (SSRT), the quantitative direct and indirect axon reflex test (QDIRT), silastic imprint test (SIT) and the dynamic sweat test (DST) are some the most used tests. While TST assesses central function, QSART, QDIRT, DST, SSRT and SIT may be used to study sweat gland activity in more detail (see Lahrmann et al, 2011, Low and Sletten, 2009, Mathias and Bannister, 2006, 2012 for a review). Limitations to the sweat tests which can affect its reproducibility and fiability are the sensitivity of the results to ambient temperature and humidity and the patient´s hydration status and caffeine intake.

II c. Invasive and biochemical techniques applied to autonomic evaluation

A good method for measuring sympathetic nervous system activity in patients is applying tests that assess individual sympathetic nervous outflows such as microneurography and measurement of norepinephrine (NE) spillover to plasma from the sympathetic nerves of individual organs (Esler *et al.*, 2003; Esler, 2011). Alternatively, global sympathetic activity may also be assessed from analysis of plasma or urine catecholamine concentrations (Goldstein *et al.*, 1983; Dimsdale & Ziegler, 1991).

Microneurography permits to separate recordings of sympathetic nerve activity to muscle (MSNA) or skin (SSNA) vessels. MSNA reflects the vasoconstrictor signal to the skeletal muscle vasculature. It is highly sensitive to BP changes and is regulated by both arterial and cardiopulmonary reflexes. These reflexes do not affect SSNA. SSNA reflects

vasomotor neural traffic to skin blood vessels with almost no sudomotor activity. The two recordings (MSNA and SSNA) differ significantly with regard to morphology. Studies to date have shown that measurement of sympathetic nerve activity from peripheral nerves is safe, accurate and reproducible. Furthermore, it has been proved that recordings from one limb can be reliably assumed to reflect recordings of sympathetic nerve activity to the muscle vascular bed throughout the body. The method's quantitative nature is also a significant advantage (Sinski *et al.*, 2006; Zygmunt & Stanczyk, 2010).

The evaluation of the activity of the SNS based on the plasma or urine NE concentration have significant limitations, as NE is subjected to changeable reuptake dependent on the density of the basilar plexus and blood flow velocity in a specific organ. Moreover, circulating NE represents only a small fraction (5–10%) of the amount of neurotransmitter secreted from nerve terminals (Sinski *et al.*, 2006). The measurement of plasma NE is, however, an improvement over the assessment of urine epinephrine (E), NE and their precursors and metabolites, which were traditionally used to evaluate the ANS tone (Sinski *et al.*, 2006; Zygmunt & Stanczyk, 2010).

Norepinephrine spillover rate has advantages over the above-mentioned, since allows the assessment of NE release from specific target organs. The NE radiolabelled method is based on intravenous infusion of small amounts of tritiated NE, which allows tissue clearance of this substance to be subtracted from plasma NE values and the remainder to be made a marker of the neurotransmitter “spillover” from neuroeffector junctions. This “spillover” in steady-state conditions mirrors the secretion of NE from the sympathetic nerve terminals (Sinski *et al.*, 2006; Zygmunt & Stanczyk, 2010). Invasive techniques measure not only total body but also regional NE spillover in the heart, splanchnic and renal circulation, and the brain (Mathias, 2003).

Experimental quantification of the activity of the SNS in animals can be undertaken using several methodologies (Grassi, 1998; Grassi & Esler, 1999; Esler *et al.*, 2003). Direct recordings of SNA (e.g. renal or lumbar) are commonly obtained in animals by the surgical implantation of recording electrodes onto the appropriate sympathetic fibres (Stocker & Muntzel, 2013).

II d. Evaluation of baroreflex function

Baroreceptor function is one of the most important regulatory mechanisms of moment to moment BP which can be evaluated through baroreflex sensitivity tests which relate the changes in heart period resulting from a change in BP. There are several methods to evaluate baroreceptor sensitivity (BRS) under dynamic or steady state conditions by using physiological or pharmacological approaches. The most common techniques to quantify BRS include pharmacological methods using vasoactive drugs (Oxford method), the Valsalva manoeuvre, the neck chamber technique and analysis of spontaneous fluctuations of BP and HR. The Oxford method uses phenylephrine (an $\alpha 1$ agonist) to induce a rapid increase in BP (15mmHg to 40mmHg) together with HR changes. Modifications of the Oxford method evaluate BRS through sequential injections of depressor and pressor drugs. There is some controversy on the usage of phenylefrine due to the selectivity of the reflex arc target as other reflex arcs, namely the chemoreceptor and the pulmonary mechano and chemoreceptors, can also be activated. Applying negative or positive pressures to the neck allows the selective activation of carotid baroreceptors and can act as an excitatory or inhibitory stimulus depending if positive or negative pressure is applied.

Computer-based techniques allow assessment of BRS by correlating spontaneous fluctuations of BP with consecutive HR changes. These computational methods may be divided into time domain, frequency domain and computational modelling (see table 3-A for detail). Time (sequence) and spectral techniques have proven reliability and have become a standard tool in many autonomic testing devices (Lahrmann *et al*.,2011).

The sensitivity of the baroreceptor reflex (BRS) can be determined by the sequential method (Di Rienzo *et al.*, 1983). This method searches ramps of blood pressure and RR. A ramp defines a variation of at least 1 mmHg and 4ms between adjacent values of BP and RR, respectively. This concept only can be applied to 3 or more cardiac cycles when they vary monotonically either increasing or decreasing. When a BP ramp occurs at the same time of a RR ramp, a BRS event is found. The sensitivity of the baroreflex can be determined by the average BRS slope: BRS =ΔRRI (ms)/ ΔsBP (mmHg) (Gratze *et al.*, 1998).

A steeper slope indicates a high sensitivity while a smaller slope indicates a lower sensitivity of the baroreflex. The baroreflex effectiveness index (BEI) is the relationship between the total number of BRS events divided by the total number of pressure ramps, increasing or decreasing, for a given period of time. BEI is an indicator of the effectiveness of baroreceptor-mediated cardiac regulation.

Table 3-A. Summary of time, frequency and modelling methodologies of BRS evaluation

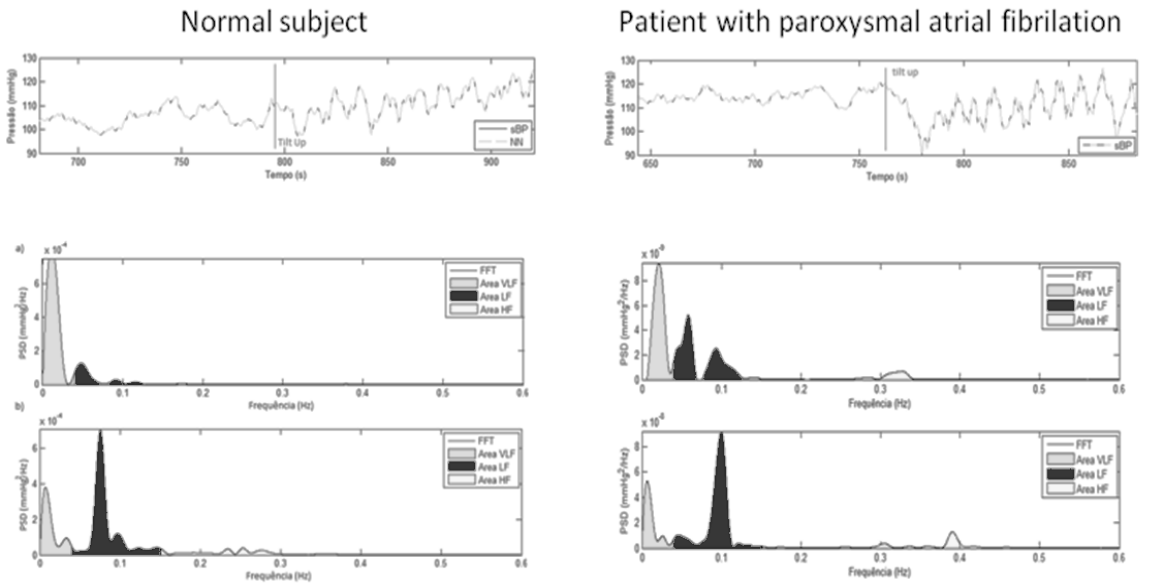
	Method	Brief description
Time domain	Sequences technique DiRienzo et al, 1985; Bertineri et al, 1988	BRS as the average of the slopes between SBP and RR values in each identified baroreflex sequence, considering SBP with one beat lag
	Dual sequence method Malberg et al, 2002	Equivalent to the sequences technique, allowing identification of baroreflex sequences considering the SBP and RR with a shift up to 3 beats
	xBRS Westerhof et al, 2004	BRS as the slope between the SBP and RR values over 10s widows choosing the shift (up to 5 beats) that maximizes the SBP and RR cross correlation. SBP and RR series resampled at 1Hz
	Events technique Gouveia et al, 2009	BRS as one global slope between the SBP and RR values in all identified baroreflex events considering SBP with one lag with respect to RR
Frequency domain	Transfer function Robbe et al, 1987	BRS as the mean value of the transfer function magnitude between SBP and RR in the LF frequency band
	Alpha technique Pagani et al, 1988	BRS as the square root of the ratio between RR and SBP powers in the LF frequency band
Model based	Closed-loop bivariate Barbieri and Saul, 1999	Quantification of the feedback feedforward SBP and RR pathways assuming a closed loop SBP and RR system
	Closed loop trivariate Barbieri et al, 1997	Quantification of the feedback feedforward SBP and RR pathways two ways pathway between SBP, RR and respiration
	xAR Porta et al, 2000	Quantification of the feedback feedforward SBP and RR pathways considering respiration as an exogenous input in the SBP and RR loop assuming a closed loop SBP and RR system
	Causal analysis Nollo et al, 2001	Quantification of the BRS assuming exogenous input model able to separate the RR variability SBP related and unrelated parts

II e. Analysis of biological signals variability

The fact that physiological signals rhythm is not entirely regular has called the attention for the possibility of extracting an autonomic signature from these signals using signal processing methods. Extremely complex neural mechanisms are responsible for these fluctuations. They are based mainly on interactions between the sympathetic and parasympathetic nervous system. So, they represent a rich source of information that can provide considerable insight into the mechanisms of cardiovascular control (Akselrod *et al.*, 1981; Malliani *et al.*, 1983; Mancia, 1983; Mancia *et al.*, 1986; Appel *et al.*, 1989; Parati *et al.*, 1992; Mancia, 1993). The cardiovascular signals, in particular HR, are the most commonly used. However, like in any biological evaluation where the environment conditions the result, standardization is still a problem mainly due to the fact that the great majority of autonomic evaluation is performed without the deep knowledge of methods and a light physiological background. At this point, confounding data are generated and misinterpretations of physiological phenomena have been drawn. Despite that, signal processing methods have been revealed, when correctly used, an important tool for the construction of autonomic markers and to the refinement of all types of therapeutics (pharmacological, surgical and others) allowing a better patients follow-up.

Signal processing can be applied, at least, in three domains, time, frequency and time – scale, each of them in an individual or complementary way being able to evidence different pathological response profiles, such as delays in the adaptive responses to the provocative manouvres, dysynergy between BP and HR responses and/or exaggerated responses such as orthostatic hypotension, postural orthostatic tachycardia or syncope (Lahrmann *et al.*, 2011; (Hilz & Dütsch, 2006).

In particular, the application of FFT and autoregressive spectral analysis to HR and BP signals has made a very important contribution to autonomic evaluation (Fig. 22-A) (Akselrod *et al.*, 1981; Akselrod *et al.*, 1985; Pomeranz *et al.*, 1985; Pagani *et al.*, 1986, Lahrmann *et al*., 2011; Hilz & Dütsch, 2006). FFT, by using sinus functions of different frequencies and amplitudes, decomposes the signals allowing a definition of a power spectrum where two major ranges of frequencies for human subjects can be recognised: very low frequencies (VLF; < 0.04Hz), low frequencies (LF; 0.04–0.15Hz) and high frequencies (HF; 0.15–0.4Hz) (M Malik, 1996).



	LF	HF	LF/HF
Basal /mmHg^2	3.0113	0.3355	8.9766
HUT/mmHg^2	13.3544	2.4059	5.5507

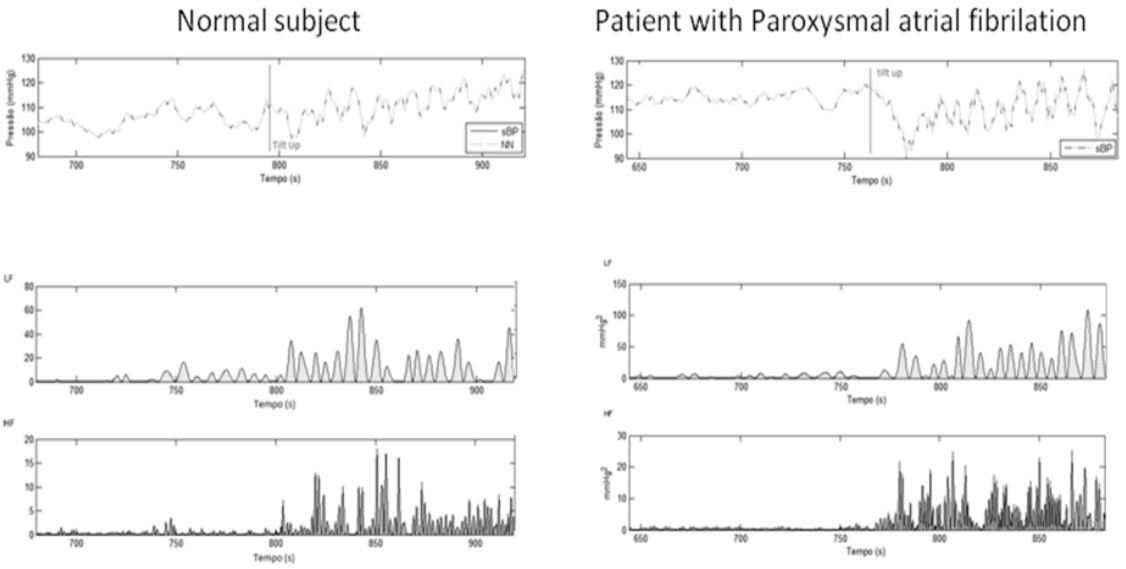
	LF	HF	LF/HF
Basal /mmHg^2	1.8338	0.3170	5.7844
HUT/mmHg^2	22.5475	3.8800	5.8113

Figure 22-A. FFT application to RRI and sBP signals from a normal subject and a patient with paroxysmal atrial fibrillation. Extracted from Oliveira et al, 2010.

The VLF band is believed to be related to non-neural factors, such as temperature and hormones (Bianchi *et al.*, 1997). The HF band is dominated by the PNS (Malik *et al.*, 1996; Akselrod *et al.*, 1997), whereas the LF band is believed to be mediated by both the cardiac sympathetic and parasympathetic nervous outflows. In rats, LF is between 0.15–0.6 Hz and the HF between 0.6–2.0 Hz (Malik *et al.*, 1996; Marques-Neves *et al.*, 2004) and for the rabbit the values are LF between 0.072 - 0.28Hz and HF between 0.29 – 1Hz (Rocha et al, 2006).

Guzzetti and co-workers reported that patients with essential hypertension are characterized by a greater LF power and a smaller HF power of RR interval during supine rest when compared with normotensive subjects (Guzzetti *et al.*, 1988). They also referred that the powers showed a smaller increase and decrease, respectively, during passive tilting. These observations were interpreted as indicating that cardiac sympathetic

tone is increased and cardiac vagal tone and modulation are decreased in essential hypertension, a conclusion that is in accordance with previous studies in which autonomic cardiac modulation was investigated by different techniques (Folkow, 1982; Julius & Johnson, 1985). FFT analysis, however, shows important limitations as it requires a stationary signal and a long period of data collection of, at least, 5 minutes and is not useful to locate and follow changes of a frequency over time. To overcome some FFT limitations, like its application to nonstationary and nonlinear signals, a wavelet based methodology was proposed to provide a time evolution of LF and HF frequencies (Fig. 23-A) (Ducla-Soares *et al.*, 2007; Laranjo et al, 2011, Postolache *et al*., 2003).



	LF	HF	LF/HF
Basal /mmHg^2	2.3344	0.2932	7.9596
HUT /mmHg^2	11.5103	2.3346	4.9306

	LF	HF	LF/HF
Basal /mmHg^2	2.4136	0.3077	7.8760
HUT/mmHg^2	23.6616	4.6263	5.1141

Figure 23-A. Wavelet analysis of RRI and SBP signals of a patient with paroxysmal atrial fibrillation compared with the same type of data analysis from a normal subject matching age and sex. Extracted from Laranjo et al, 2011.

Wavelet analysis is a linear and nonstationary representation method of signals in time and frequency domains. For the wavelet analysis it is necessary to have a basic function, called mother wavelet, which allows the decomposition of the original signal in

translational versions of this function with different base scale. A mother wavelet function is a nonperiodic, oscillatory function that begins and ends at zero in time domain (Kaiser & SpringerLink, 2011). However, being a good alternative to FFT, wavelets (WT) lack in resolution, particularly in low frequencies.

The Hilbert Transform (HT) is a linear operator able to determine the instantaneous frequency of a signal, corresponding to the convolution of the input signal with the kernel. In order to reach a physiological meaning for the amplitude, the frequency and the phase, the signal to be transformed must have an instantaneous null DC component (Huang *et al.*, 1998).

Recently, Huang proposed to fulfil this condition through the Empirical Mode Decomposition (EMD) applied to nonlinear and nonstationary processes. The combination of Hilbert Transform with the EMD has resulted in what is known as the Hilbert-Huang Transform (HHT) (Fig. 24-A and 25-A). In our laboratory, we developed an integrated and modular system - FisioSinal® - for clinical and laboratorial autonomic evaluation using cardiovascular signals.

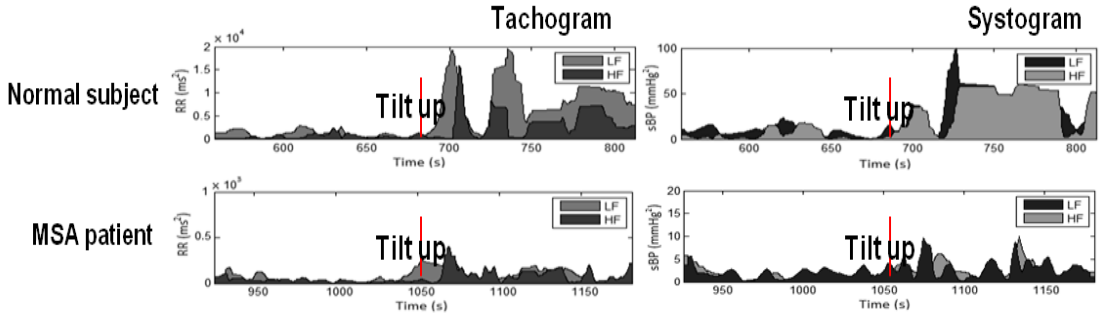
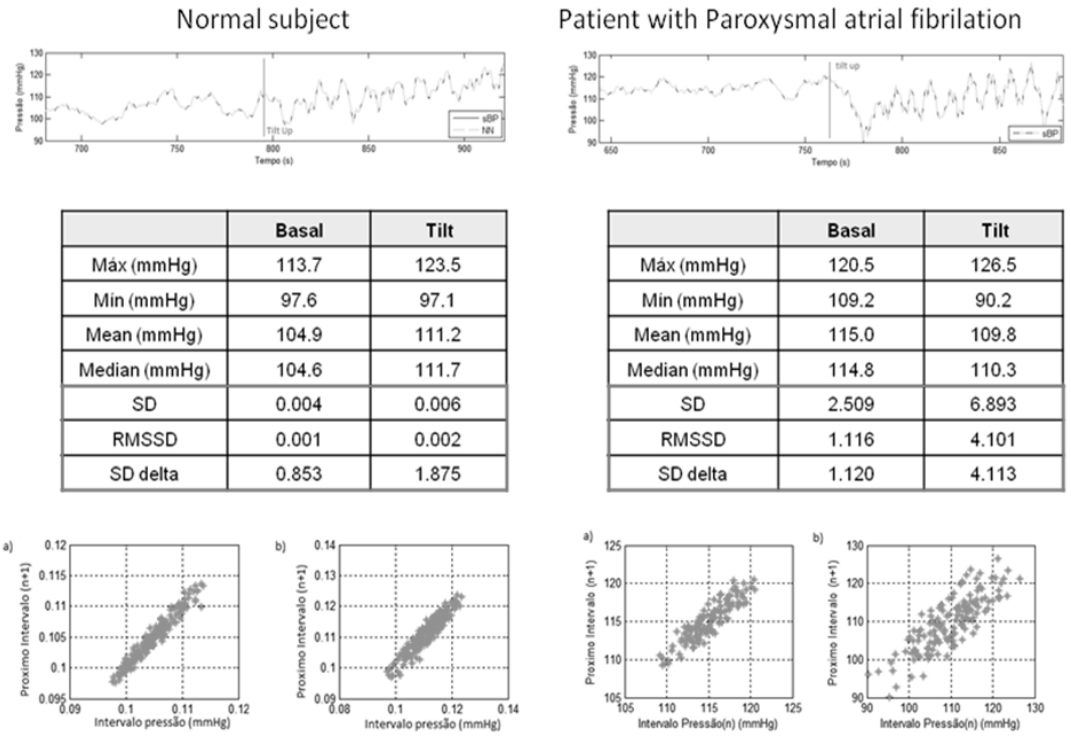


Figure 24-A. RRI and BP recorded during an HUT of a patient with multiple system atrophy (MSA) where analysed using HHT. Data are compared with a response of a normal individual matching sex and age. MSA is characterised by a strong disautonomy as shown by HHT analysis. Extracted from Tavares et al, 2010.

The computational tools that are included in FisioSinal are: statistics (Fig. 25-A), fast Fourier transform, Wavelet and Hilbert-Huang transforms, baroreflex effectiveness index and wavelets coherence (Fig. 26-A) (Tavares, 2011a; Tavares *et al.*, 2012)



	Basal	Tilt
Máx (mmHg)	113.7	123.5
Min (mmHg)	97.6	97.1
Mean (mmHg)	104.9	111.2
Median (mmHg)	104.6	111.7
SD	0.004	0.006
RMSSD	0.001	0.002
SD delta	0.853	1.875

	Basal	Tilt
Máx (mmHg)	120.5	126.5
Min (mmHg)	109.2	90.2
Mean (mmHg)	115.0	109.8
Median (mmHg)	114.8	110.3
SD	2.509	6.893
RMSSD	1.116	4.101
SD delta	1.120	4.113

Figure 25-A. The statistical methods are also used for autonomic evaluation. This figure shows the statistical analysis of RRI and SBP values showed through a Poincaré plot (Tavares et al, non published observations).

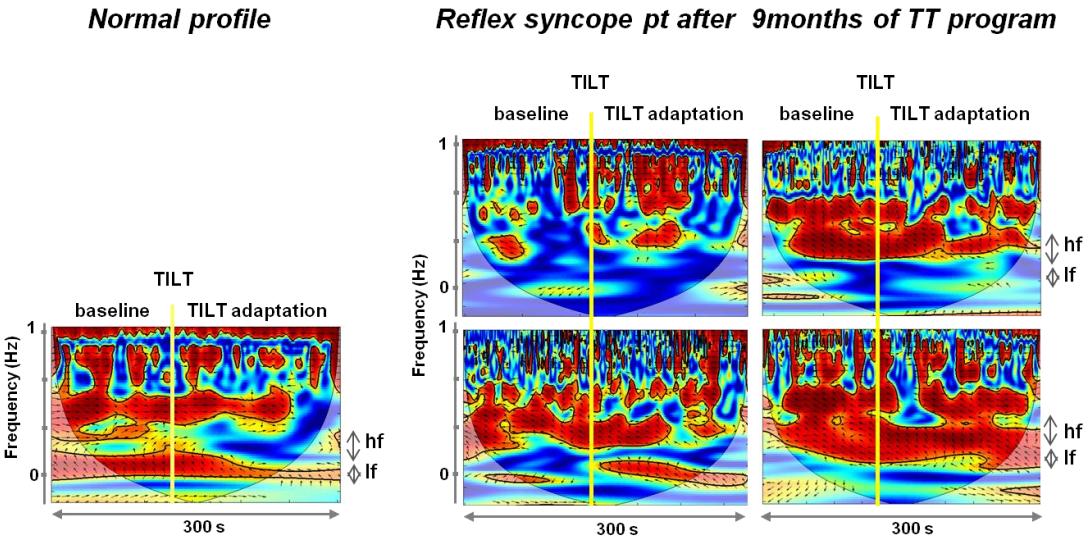


Figure 26-A. Autonomic analysis tools used to show reverse autonomic modulation in patients with reflex syncope. On the left, are depicted the changes in wavelets coherence evoked by a tilt maneuver in a normal subject. After tilting (vertical line) there is a drop of coherence, which reaches its minimum value approximately 20 s after tilting, recovering later to a significant lower value. On the right, modification of HR and SBP variability coherence along a tilt training period used to induce autonomic remodeling n

patients with reflex syncope (A: basal conditions before training program; B, C and D: 1st, 4th and 9th tilt-training sessions, respectively). The increase of coherence along the training sessions, which relates with an increase of baroreceptor remodeling, is represented by an improvement of the band organization together with a higher density of the orange/red color. These graphic changes are better seen after tilting-up (vertical line). Extracted from Laranjo *et al.*, 2014.

APPENDIX 2

Experimental Physiology

Research Paper

Chronic depression of hypothalamic paraventricular neuronal activity produces sustained hypotension in hypertensive rats

Vera Geraldes[1], Nataniel Gonçalves-Rosa[1], Beihui Liu[2], Julian F. R. Paton[2] and Isabel Rocha[1,3]

[1]*Cardiovascular Autonomic Function Laboratory, Faculty of Medicine and Instituto de Medicina Molecular and* [3]*Centro de Cardiologia, University of Lisbon, Portugal*

[2]*School of Physiology & Pharmacology, Bristol Heart Institute, Medical Sciences Building, University of Bristol, Bristol BS8 1TD, UK*

New Findings

- **What is the central question of this study?**
  Will a chronic reduction of neuronal excitability within the paraventricular nucleus of the hypothalamus reduce arterial blood pressure and sympathetic activity in the long term in an animal model of neurogenic hypertension?
- **What is the main finding and its importance?**
  We show, for the first time, that overexpression of an inwardly rectifying potassium channel in the paraventricular nucleus provided a long-term (>60 days) antihypertensive response in conscious spontaneously hypertensive rats that was associated with a reduction in neurohumorally mediated vasoconstriction, enhanced baroreflex sensitivity and reduced peripheral chemosensitivity; no such response was observed in normotensive rats. Our results support the paraventricular nucleus as a therapeutic target for the chronic control of blood pressure in neurogenic hypertension.

Changes in the sympathetic nervous system are responsible for the initiation, development and maintenance of hypertension. An important central sympathoexcitatory region is the paraventricular nucleus (PVN) of the hypothalamus, which may become more active in hypertensive conditions, as shown in acute studies previously. Our objective was to depress PVN neuronal activity chronically by the overexpression of an inwardly rectifying potassium channel (hKir2.1), while evaluating the consequences on blood pressure (BP) and its reflex regulation. In spontaneously hypertensive rats (SHRs) and Wistar rats (WKY) lentiviral vectors (LVV-hKir2.1; LV-TREtight-Kir-cIRES-GFP5 4×10^9 IU and LV-Syn-Eff-G4BS-Syn-Tetoff 6.2×10^9 IU in a ratio 1:4) were stereotaxically microinjected bilaterally into the PVN. Sham-treated SHRs and WKY received bilateral PVN microinjections of LVV-eGFP (LV-Syn-Eff-G4BS-Syn-Tetoff 6.2×10^9 IU and LV-TREtight-GFP 5.7×10^9 IU in a ratio 1:4). Blood pressure was monitored continuously by radio-telemetry and evaluated over 75 days. Baroreflex gain was evaluated using phenylephrine (25 μg ml^{-1}, I.V.), whereas lobeline (25 μg ml^{-1}, I.V.) was used to stimulate peripheral chemoreceptors. In SHRs but not normotensive WKY rats, LVV-hKir2.1 expression in the PVN produced time-dependent and significant decreases in systolic (from 158 ± 3 to 132 ± 6 mmHg; $P < 0.05$) and diastolic BP (from 135 ± 4 to 113 ± 5 mmHg; $P < 0.05$). The systolic BP low-frequency band was reduced (from 0.79 ± 0.13 to 0.42 ± 0.09 mmHg^2; $P < 0.05$), suggesting reduced sympathetic vasomotor tone. Baroreflex gain was increased and peripheral chemoreflex depressed after PVN microinjection of LVV-hKir2.1. We conclude that the PVN plays a major role in long-term control of BP and sympathetic nervous system activity in SHRs.

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This is associated with reductions in both peripheral chemosensitivity and respiratory-induced sympathetic modulation and an improvement in baroreflex sensitivity. Our results support the PVN as a powerful site to control BP in neurogenic hypertension.

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Corresponding author I. Rocha: Instituto de Fisiologia, Faculdade de Medicina de Lisboa, Av Prof Egas Moniz, 1649-028 Lisbon, Portugal. Email: isabelrocha@fm.ul.pt

Introduction

Essential arterial hypertension has now reached pandemic proportions, with an estimated one billion sufferers worldwide. The pathogenesis of essential arterial hypertension is multifactorial and not completely understood, but there is clear evidence that chronic elevation of sympathetic nervous system activity is a major contributor to the onset, development and maintenance of the hypertensive state (Grassi, 2004*b*; Guyenet, 2006; Fisher & Paton, 2012). In fact, the increase of sympathetic outflow to the heart results in increased cardiac output and neurally mediated vasoconstriction, leading to elevated blood pressure values (Schlaich *et al.* 2012). In 'white coat' and borderline hypertensive patients, sympathetic nerve activity to the arterioles supplying skeletal muscle is raised in comparison to healthy individuals (Grassi, 2004*a*; Smith *et al.* 2004). Excessive sympathetic activity may contribute to hypertrophy of vascular smooth muscle and cardiac muscle, brain hypoperfusion and inflammation, and becomes a major target to control in neurogenic hypertension (Zubcevic *et al.* 2011).

The evaluation of sympathetic activity can be achieved indirectly by applying mathematical tools such fast Fourier transform to blood pressure signals (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). A power spectrum is generated, where the low frequencies (LFs) represent predominantly sympathetic activity and high frequencies (HFs) are related to parasympathetic tonus and respiration (Radaelli *et al.* 1994; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Furlan *et al.* 2000). These mathematical results have been addressed in several studies, which have suggested that sympathetic activity is a critical determinant of blood pressure fluctuations in a frequency range which is slower than the rate of respiration (Japundzic *et al.* 1990; Cerutti *et al.* 1991; Malliani *et al.* 1991).

Located in the hypothalamus, the paraventricular nucleus (PVN) is a major sympathoexcitatory area that becomes more active in conditions of hypertension, such as in the spontaneously hypertensive rat (SHR) model (Allen, 2002). Some authors have referred to this region as a command nucleus providing feedforward excitatory synaptic drives to co-ordinate lower brainstem cardiovascular and respiratory motor activity (Dampney *et al.* 2005). Activation of the PVN promotes an increase in sympathetic output and a pressor effect mediated via direct and indirect projections (via the rostral ventrolateral medulla) to the spinal cord (Caverson *et al.* 1984; Shafton *et al.* 1998; Pyner & Coote, 2000; Hardy, 2001).

Both electrical stimulation and chemical manipulation of PVN neurons with bicuculline (a $GABA_A$ receptor antagonist) or glutamate elevated sympathetic nerve activity and caused hypertension in anaesthetized and conscious rats (Kannan *et al.* 1989; Zhang *et al.* 2002). In contrast, acute inhibition of the PVN with GABA or muscimol reduces the blood pressure and sympathetic nerve activity in SHRs (Allen, 2002). Lesions of the PVN or transection of the brain caudal to the hypothalamus promotes a decrease in blood pressure in SHRs but not in Wistar–Kyoto (WKY) rats (Yamori & Okamoto, 1969; Goto *et al.* 1981; Ciriello *et al.* 1984; Herzig *et al.* 1991; Takeda *et al.* 1991).

Long-term manipulation of neurone excitability can be performed by expressing a human inwardly rectifying potassium channel (hKir2.1) under the control of a selective neuronal promoter, such as synapsin (Duale *et al.* 2005, 2007). Inwardly rectifying potassium channels, such as Kir2.1, are endogenously expressed in rat brain and have recently been overexpressed as a means to reduce neuronal membrane excitability (Yu *et al.* 2004; Duale *et al.* 2007; Mizuno *et al.* 2007; Okada & Matsuda, 2008; Yoon *et al.* 2008; Howorth *et al.* 2009). Their long-term expression can be achieved by the use of lentiviral vectors (LVVs) derived from human immunodeficiency virus (Coleman *et al.* 2003). Therefore, using a LVV to overexpress hKir2.1 channels within the PVN, we sought to determine the long-term influence of this nucleus on the control of blood pressure, heart rate, sympathetic activity and respiration in SHRs, as well as homeostatic reflex control mechanisms.

Methods

All the experimental procedures were in accordance with the European and Portuguese Law on animal welfare and had the approval of the ethics committee of the Faculty of Medicine, University of Lisbon, Portugal. Male Wistar–Kyoto rats ($n = 15$) and SHRs ($n = 15$) were used, aged 12 weeks and weighing 363 ± 8 g. Animals, synchronized to a 12 h–12 h light–dark cycle (light on at 07.00 h and light off at 19.00 h), were housed individually and allowed to freely move in standard plastic cages. Food and water were available *ad libitum*.

Viral vector construction and validation

Lentiviral vector construction was based on previous studies (Waki *et al.* 2003; Duale *et al.* 2007). Briefly, LVV-eGFP, used for the sham-treated group, was a mix of LV-TREtight-GFP 5.7×10^9 IU and LV-Syn-Eff-G4BS-Syn-Tetoff 6.2×10^9 IU in a ratio 1:4. This binary system expresses enhanced green fluorescent protein (eGFP). The LVV-hKir2.1 is a mix of LV-TREtight-Kir-cIRES-GFP 5.4×10^9 IU and LV-Syn-Eff-G4BS-Syn-Tetoff 6.2×10^9 IU in a ratio 1:4, which expresses eGFP and expresses human inwardly rectifying potassium channels (hKir2.1) in neurones. Validation of transduction efficacy and transgene expression was assessed as described previously by Duale *et al.* (2007) and included mRNA expression, immunocytochemical and electrophysiological data.

Microinjection sites

Initially, we fine tuned our stereotaxic co-ordinates for bilateral PVN microinjections in five SHRs and five WKY rats anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, I.P., Hikma Pharmaceuticals, London, UK). Bilateral microinjections (0.05 μl) of LVV-eGFP were performed. Using fluorescence microscopy and histological reconstruction, we determined the correct co-ordinates for PVN injections and the amount of LVV-eGFP needed to limit transduction to the confines of the PVN.

Surgery

Spontaneoulsy hypertensive rats were divided into two groups according to the microinjection content, i.e. LVV-hKir2.1 ($n = 8$) and LVV-eGFP ($n = 7$). A control group of WKY rats, with matching age, sex and number of individuals, underwent the same surgical and experimental protocol.

Implantation of telemetry probes. Rats were implanted with radio-telemetry probes (DSI, St. Paul, Minnesota, MN, USA) in the abdominal aorta under general anaesthesia (sodium pentobarbitone, 60 mg kg^{-1}, I.P., Hikma Pharmaceuticals). Animals were allowed to recover for 15 days. Similar anaesthetic and surgical protocols were applied to WKY rats ($n = 15$).

Bilateral microinjection in the PVN. Two weeks after the probes were implanted, SHRs ($n = 8$) and WKY rats ($n = 8$) under general anaesthesia (sodium pentobarbitone, 60 mg kg^{-1}, I.P., Hikma Pharmaceuticals) were placed in a stereotactic frame (Kopf Instruments, Tujunga, CA, USA), and a craniotomy was performed using our previously determined co-ordinates for LVV-hKir2.1 microinjections (0.05 μl) into the PVN (Bregma, −1.6 mm; Lateral, ±1.41 mm; Deep, 7.4 mm; pipette angle, 10 deg to bregma; Paxinos & Watson, 1986). Sham-treated rats were microinjected in the same region with LVV-eGFP (SHRs, $n = 7$; and WKY rats, $n = 7$). All microinjections were performed bilaterally. Animals of all groups were allowed to recover and monitored by telemetry for 60 days. Heart rate (HR) and blood pressure [BP; systolic (SBP), diastolic and mean] were recorded continuously.

Metabolic evaluation

Rats were housed for 24 h in metabolic cages to evaluate body weight, intake of food and fluid and production of urine and faeces. Measurements were performed before and 59 days after each microinjection.

Cardiorespiratory reflex evaluation

At 60 days, animals were anaesthetized (sodium pentobarbitone, 60 mg kg^{-1}, I.P., Hikma Pharmaceuticals). The trachea was cannulated below the larynx to record tracheal pressure. The femoral and carotid arteries and femoral vein were cannulated. Rectal temperature was maintained at 38 ± 1°C by a servo-controlled heating blanket. The ECG was recorded with the use of needle electrodes inserted into the limbs, and HR was derived from the ECG. Baroreceptor and peripheral chemoreceptor reflexes were activated twice, with an interval of 5 min between stimuli. Baroreceptor reflex was stimulated by phenylephrine (0.2 ml, 25 μg ml^{-1} I.V.; Sigma Aldrich). Peripheral chemoreceptor reflex was stimulated with lobeline (0.2 ml, 25 μg ml^{-1}, Sigma Aldrich) injected retrogradely into the bifurcation of the common carotid artery. Heart rate, BP (systolic, diastolic and mean) and respiratory rate (RespR) were recorded continuously thought the experiment.

Histology and immunochemistry

Animals were terminally anaesthetized and immediately perfused transcardially with PBS (0.1 M; pH 7.4) followed by 4% paraformaldehyde (0.1 M; pH 7.4). The brain was removed and placed for 48 h in 15% (w/v) sucrose solution. Coronal sections (18 μm thick) were cut on a microtome and mounted on slides. The pipette tip location and the microinjection diffusion in the PVN were examined and documented. The microinjected contents (LVV-hKir2.1 or LVV-eGFP) containing eGFP allowed an estimation of virus dispersion. The eGFP-labelled fluorescent regions were identified using an epifluorescence microscope and plotted on standardized sections from the atlas of Paxinos & Watson (1986).

Western blot analysis

The expression of hKir2.1 in the PVN was analysed by Western blot 60 days after the microinjection of

LVV-hKir2.1 ($n = 8$) or LVV-eGFP in SHRs ($n = 7$). The PVN was dissected from both groups and homogenized by sonication in ice-cold RIPA buffer (Sigma, St. Louis, MO, USA) supplemented with a cocktail of protease inhibitors (complete mini; Roche). Proteins were extracted from the homogenates by centrifugation at 5000*g* for 10 min at 4°C, and protein concentration was determined with a Bio-Rad DC Protein Assay kit. Proteins were resolved by electrophoresis on a 10% Tris–glycine SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5% milk in Tween/Tris-buffered saline and incubated overnight at 4°C with rabbit anti-hKir2.1 polyclonal antibody (Abcam, Cambridge, UK). After washing, membranes were incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit antibody (Bio-Rad, Hercules, CA, USA), and immunoreactive proteins were detected by Immobilon Western Chemiluminescent HRP Substrate (Millipore, Bedford, MA, USA) and visualized using Curix 60 (AGFA, Greenville, SC, USA). Membranes were stripped with 0.1 M glycine (pH 2.2) and reprobed with the α-tubulin antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for loading control.

Data acquisition and analysis

Telemetric data were acquired at 1 kHz and analysed with suitable software (LabChart6, Powerlab; ADInstruments, Oxford, UK). Mean values of HR, BP (systolic, diastolic and mean) and RespR were extracted.

Baroreceptor and chemoreceptor reflex. The baroreceptor reflex gain (BRG) was quantified by calculating ΔHR/ΔBP (in beats per minute per millimetre of mercury). Chemoreceptor (ChR) reflex was calculated through the RespR derived from the tracheal pressure before and after stimulation with lobeline, as follows: $\Delta ChR = RespR_{lobeline} - RespR_{basal}$. Blood pressure and HR were also evaluated.

Analysis of BP and HR variability. Systolic BP and R–R interval data were analysed (period of 3 min) in the frequency domain (Fast Fourier Transform), using the in-house software Fisiosinal (Tavares, 2011), to evaluate sympathetic (LF band, 0.15–0.6 Hz of systolic BP) and parasympathetic activity (HF band, 0.6–2.0 Hz of HR) over time (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Marques-Neves *et al.* 2004).

Circadian light/dark heart rate and blood pressure profile. Mean BP and HR values were calculated using the continuous telemetric data and compared between light (07.00–19.00 h) and dark phases (19.00–07.00 h).

Statistical analysis

Comparisons were performed between groups for the same period and within the same group, before and after the microinjections. For the statistical analysis, Student's paired *t* test and ANOVA for comparisons between groups were used. All data were expressed as means ± SEM and passed the normality test. Significance was taken as $P < 0.05$.

Results

Effect of microinjection of LVV-hKir2.1 or LVV-eGFP on 24 h mean values of blood pressure, heart rate and respiration

Basal BP values (recorded before microinjections) in conscious SHRs ($n = 15$) were 158 ± 3 mmHg for systolic BP, 135 ± 4 mmHg for diastolic BP and 142 ± 3 mmHg for mean BP, and were significantly higher than the values for WKY rats (119 ± 3, 91 ± 2 and 101 ± 1 mmHg, respectively; $n = 15$; $P < 0.0001$). The SHRs showed a higher baseline respiratory rate than WKY rats (77 ± 5 *versus* 61 ± 4 breaths min^{-1}, respectively; $P < 0.05$) as well as a lower HR (311 ± 5 and 367 ± 9 beats min^{-1}; $P < 0.0001$).

Thirty days after LVV-hKir2.1 microinjection, a significant BP decrease ($P < 0.05$) was first observed, but in order to evaluate its persistence, animals were monitored for a further 30 days. On the 60th day after lentiviral microinjection, values for SHRs for systolic, diastolic and mean BP were 132 ± 6, 113 ± 5 and 120 ± 5 mmHg, respectively, corresponding to a decrease in pressure of 26, 22 and 22 mmHg, respectively ($P < 0.01$; Fig. 1). These BP changes were accompanied by a lowering of HR (295 ± 3 beats min^{-1}, $P = 0.099$) but RespR remained unchanged. The decreased BP and HR values approached those of normotensive animals. At the same time, SHRs microinjected with LVV-eGFP were showing increased values of systolic (174 ± 10 mmHg; $P > 0.05$), diastolic (149 ± 11 mmHg; $P > 0.05$) and mean BP (157 ± 10 mmHg; $P > 0.05$), together with a significantly decreased HR (285 ± 6 beats min^{-1}; $P < 0.01$). This profile of BP and HR changes was expected and is a consequence of maturation. In contrast, no significant changes in BP, HR and RespR were observed in WKY rats during the 60 day duration of the experimental protocol.

Effect of LVV-hKir2.1 microinjection on sympathetic output measured indirectly

Spontaneously hypertensive rats showed putative evidence for an overall decrease of cardiovascular autonomic outflow at 60 days after LVV-hKir2.1 microinjection when compared with basal autonomic output at day 0. In fact,

by using fast Fourier transform applied to SBP and R–R intervals, a decrease of LF_{SBP}/HF_{RR} ratio (from 0.07 ± 0.02 to 0.04 ± 0.01 mmHg^2 ms^{-2}; $P > 0.05$) was observed, mainly due to a strong decrease in sympathetic output expressed by LF_{SBP} band power (from 0.79 ± 0.13 to 0.42 ± 0.09 mmHg^2; $P < 0.05$). In SHRs, the basal HF_{SBP} (0.75 ± 0.10 mmHg^2) was first reduced at 40 days and persisted until 60 days (0.33 ± 0.10 mmHg^2; $P < 0.05$) after LVV-hKir2.1, but it was unchanged in the LVV-eGFP group (0.82 ± 0.38 mmHg^2). Interestingly, LF SBP was significantly reduced by 20 days after LVV-hKir2.1 microinjection and occurred before the fall in SBP. In contrast, at 60 days the LF_{SBP}/HF_{RR} ratio for SHR LVV-eGFP was 0.08 ± 0.03 mmHg^2 ms^{-2} and the LF was 0.86 ± 0.21 mmHg^2 ($P > 0.05$). The variations of mean LF_{SBP} and LF_{SBP}/HF_{RR}, at 10 day intervals for each SHR group, are depicted in Fig. 2. For WKY rats in basal conditions, the LF_{SBP} and LF_{SBP}/HF_{RR} ratio were 3.23 ± 0.36 mmHg^2 and 0.43 ± 0.14 mmHg^2 ms^{-2}, respectively. No significant changes in LF and LF_{SBP}/HF_{RR} ratio were observed for WKY LVV-hKir2.1 (3.11 ± 0.44 mmHg^2 and 0.40 ± 0.23 mmHg^2 ms^{-2}, respectively) and WKY LVV-eGFP rats (2.56 ± 0.48 mmHg^2 and 0.22 ± 0.08 mmHg^2 ms^{-2}, respectively).

Arterial baroreflex gain and peripheral chemoreflex responsiveness

The injection of phenylephrine triggered, in all animal groups, a progressive increase in mean BP, which was accompanied by a progressive reduction in HR. In SHRs, BRG increased significantly after LVV-hKir2.1 microinjection and approached the values of the normal control rats. The SHR LVV-hKir2.1 group had a higher BRG than the SHR LVV-eGFP group (0.51 ± 0.06 *versus* 0.33 ± 0.03 beats min^{-1} mmHg^{-1}, respectively; $P < 0.05$; Fig. 3). Interestingly, the BRG of WKY LVV-hKir2.1 rats (1.29 ± 0.18 beats min^{-1} mmHg^{-1}) was also increased in comparison to the WKY LVV-eGFP group (0.41 ± 0.02 beats min^{-1} mmHg^{-1}; $P < 0.0001$), despite all cardiovascular variables remaining unchanged.

Respiratory rate remained unchanged throughout the full experimental protocol in all animal groups, before and after the lentiviral microinjection. At 60 days after microinjection, the baseline values of respiratory rate in the anaesthetized animals were 76 ± 3.4, 81 ± 4.9, 80 ± 4.5 and 67 ± 3.5 breaths min^{-1}, respectively, for SHR and WKY LVV-hKir2.1, SHR and WKY LVV-eGFP. However, peripheral chemoreceptor reflex activation with lobeline elicited a hyperventilatory reflex response of different magnitude according to the animal group. The SHR LVV-hKir2.1 animals showed a decreased ventilatory response when compared with the SHR LVV-eGFP group ($\Delta 24.4 \pm 3.4$ *versus* $\Delta 38.1 \pm 4.9$ breaths min^{-1}, respectively; $P < 0.05$; Fig. 3). In contrast, there were no differences in the ventilatory response between WKY LVV-hKir2.1 and WKY-eGFP groups ($\Delta 23.3 \pm 5.9$ breaths min^{-1} for WKY LVV-hKir2.1 and $\Delta 24.8 \pm 4.2$ breaths min^{-1} for WKY LVV-eGFP). Mean BP responses to chemoreflex activation in SHR LVV-hKir2.1 animals (from 140 ± 7 to 154 ± 9 mmHg) were depressed compared with SHR LVV-eGFP rats (179 ± 9 to 193 ± 9 mmHg; $P < 0.05$), but HR responses were not different (from 337 ± 23 to 359 ± 12 *versus* from 373 ± 10 to 362 ± 13 beats min^{-1}, respectively). For the two WKY groups, changes in BP and HR in response to peripheral chemoreflex activation were not different.

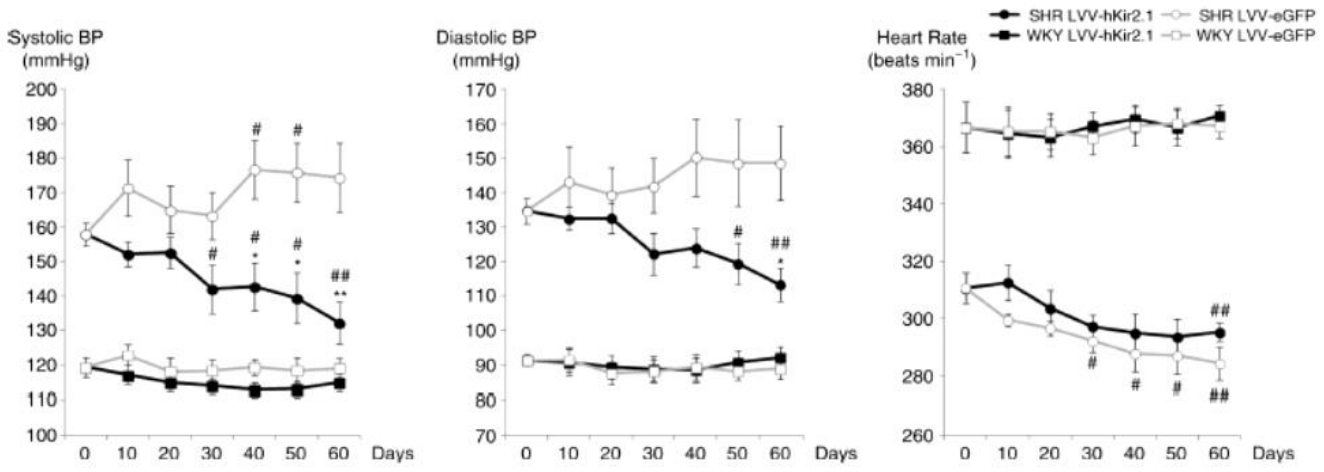

Figure 1. Effect on systolic blood pressure, diastolic blood pressure and heart rate before (0 days) and after microinjection of LVV-hKir2.1 ($n = 7$) or LVV-eGFP ($n = 7$)
*$P < 0.05$, **$P < 0.01$, statistically significant differences between spontaneously hypertensive rat (SHR) LVV-hKir2.1 and SHR LVV-eGFP groups. #$P < 0.05$, ##$P < 0.01$, statistically significant differences within the group.

Circadian variation of BP and HR and patterns of nocturnal blood pressure profile

In basal conditions and without any intervention, the pattern of circadian variation of BP and HR followed a similar trend, with lower BP values during the light phase relative to the dark phase. During the light phase, the systolic, diastolic and mean BP of SHRs were significantly higher than those for WKY rats (Table 1; $P < 0.0001$) over the same time period. The same type of variation was found for the dark phase, during which SHRs showed higher values for BP parameters than WKY rats ($P < 0.0001$; Table 1). Mean basal HR followed these variations in BP inversely. The HR was significantly lower during the light and dark phases for SHRs than for WKY rats ($P < 0.01$; Table 1).

At 60 days after the LVV-hKir2.1 microinjection, SHRs showed a significant decrease of systolic, diastolic and mean BP during both the light and the dark phase (both $P < 0.01$; Table 1). A significant decrease of HR was observed during the light but not during the dark phase ($P > 0.05$). For the SHR LVV-eGFP rats, HR, diastolic, systolic and mean BP values for the light phase and dark phase were increased as expected at 60 days (Table 1). Finally, in WKY LVV-hKir2.1 as well as WKY LVV-eGFP rats there was an increase in BP during the dark phase without a distinct circadian rhythm. This profile was maintained at 60 days after LVV-hKir2.1 and LVV-eGFP PVN microinjections (Table 1).

Metabolic evaluation

A significant decrease in food intake was observed in the SHR LVV-hKir2.1 group at 60 days after the microinjection (Table 2). No other significant changes were found in body weight, water intake, faeces and urine production for all groups, before and after the microinjections, suggesting that the physical inactivity due to social isolation (only one animal per cage) could have an impact on food consumption. Furthermore, animals were not subjected to an adaptation period to the metabolic cages, which could impact on our metabolic data, constituting a study limitation.

Histological, immunohistochemical and Western blot analysis

The microinjection sites were located within the PVN according to the rat atlas of Paxinos & Watson (1986). Enhanced green fluorescent protein was detected by fluorescence microscopy as fluorescence confined to a surface of 0.10–0.20 mm around the injection site. The eGFP did not penetrate the third ventricular ependymal lining. Through immunohistochemical studies, it was confirmed that PVN neurones expressed eGFP (Fig. 4).

The overexpression of hKir2.1 in the PVN was analysed using Western blot. The PVN dissected from SHRs microinjected with LVV-hKir2.1 showed an increased expression of hKir2.1, on average about ninefold increased when compared with the LVV-eGFP group (Fig. 4).

Discussion

In the present study, we investigated the effect of overexpressing an inwardly rectifying potassium channel

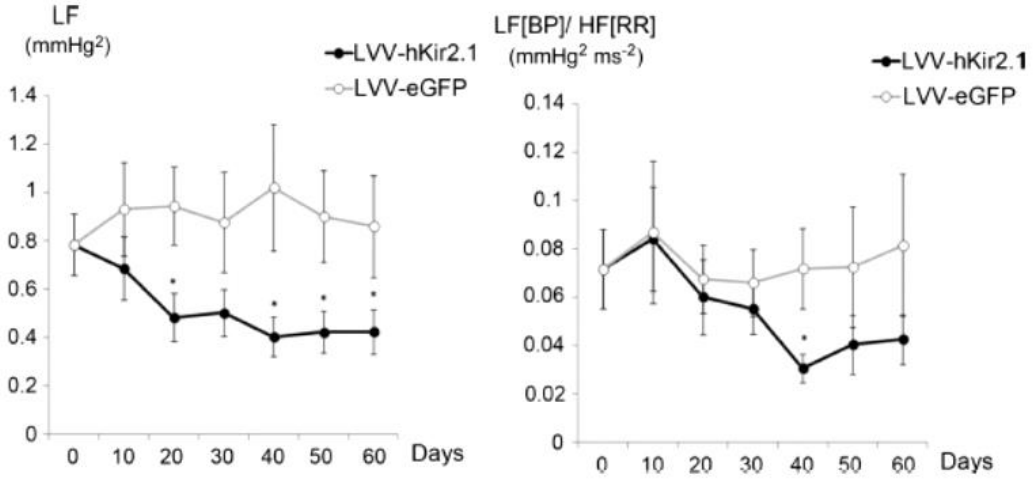

Figure 2. Mean (±SEM) low frequency (LF) and ratio of low (blood pressure) to high frequency (R–R interval) [LF(BP)/HF(RR)] before (0 days) and at 10 day intervals after the microinjection of LVV-hKir2.1 or LVV-eGFP in SHRs
Note that the fall in LF systolic blood pressure (SBP) occurred a week before the fall in SBP, suggesting a causative association. $^*P < 0.05$, statistically significant difference between groups.

in the PVN to lower neuronal activity, while measuring BP chronically, as well as its reflex control, in a rat model of hypertension. Our study is the first to demonstrate that chronic suppression of PVN neuronal activity in freely moving SHRs causes a sustained reduction in arterial blood pressure (>60 days) together with a decrease of sympathetic activity, a downregulation of peripheral chemoreflex responsiveness and an improvement of baroreflex gain. No such changes were found in the control groups of both rat strains that underwent comparable experimental protocols.

The PVN of the hypothalamus is well known for its importance in autonomic control and, in particular, for cardiovascular regulation. Several anatomical and electrophysiological studies have shown that PVN neurones project either directly to the spinal cord or to the rostral ventrolateral medulla (Coote, 2007), thereby accessing sympathetic neurones to modulate blood pressure (Hosoya *et al.* 1991; Loewy, 1991; Coote, 1995, 2005; Ranson *et al.* 1998; Motawei *et al.* 1999; Pyner & Coote, 1999, 2000; Badoer, 2001). As an example, electrolytic lesions of the PVN in SHRs elicited an acute reduction of sympathetic activity together with a decrease of blood pressure (Takeda *et al.* 1991). Other acute studies, performed under general anaesthesia, showed that muscimol injections into the PVN lowered BP and renal sympathetic nerve activity in both SHRs and WKY rats, indicating that this region was tonically active in both animal strains to control BP and peripheral sympathetic activity (Allen, 2002). In the SHR, sympathetic activity is known to be overactivated even before hypertension develops (Simms *et al.* 2009). Several studies have pointed out that the persistent increase in sympathetic tone is a major contributor to both the initiation and the maintenance of the hypertensive condition (Yamada *et al.* 1988; Grassi, 2004*b*; Smith *et al.* 2004; Guyenet, 2006; Fisher & Paton, 2012). In fact, increased sympathetic activity has been detected in normotensive individuals with a family history of hypertension and in individuals with essential hypertension, but not in those with secondary hypertension (Yamada *et al.* 1988; Grassi *et al.* 1998; Grassi, 2004*a*, 2009). Likewise, high plasmatic noradrenaline levels have also been associated with essential hypertension, being consistently increased in younger hypertensive patients (Grassi, 1998), and increased peripheral sympathetic nervous activity has been detected by microneurographic techniques in hypertensive patients (Anderson *et al.* 1989; Grassi, 1998; Greenwood *et al.* 1999; Mano, 2012). Several studies, both in human subjects and in animal models, have demonstrated an association between the circadian variation of BP values, the hypertensive condition, the sympathetic activation, the end-organ damage and the worsening of cardiovascular outcome (White, 2000; Pickering & Kario, 2001; Weber, 2002). Thus, the idea of a long-term modulation of the level of sympathetic activity, at its central origin, as a way to control and treat high blood pressure and to increase cardiovascular compliance, is very appealing. In particular, the manipulation of sympathetic cell excitability by modulation of K^+ channel expression, to hyperpolarize neuronal resting membrane potential, is an attractive hypothetical therapeutic strategy (Duale *et al.* 2007).

In the present work, our purpose was to depress the activity of PVN neurones chronically by the overexpression of K^+ channels exclusively in PVN

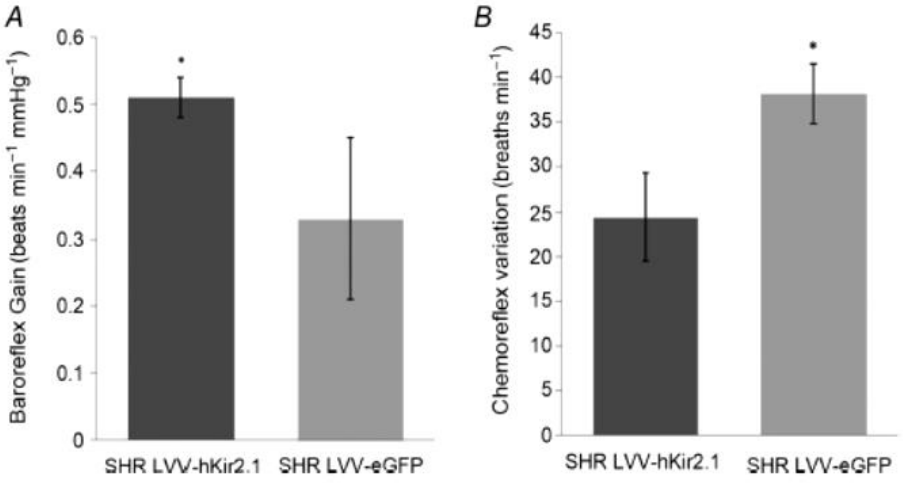

Figure 3. Effect of bilateral microinjections of LVV-hKir2.1 or LVV-eGFP into the paraventricular nucleus on baroreflex gain (*A*) and chemoreflex variation (*B*), 60 days after microinjection
In the SHR LVV-hKir2.1 group, there is an increase in the baroreflex gain and a decrease in the chemoreflex ventilatory response. *$P < 0.05$, statistically significant differences between groups.

Table 1. Blood pressure (in millimetres of mercury) and heart rate (in beats per minute) during the light and dark phases for all groups before and 59 days after the microinjection

	Light phase				Dark phase			
Group	SBP	DBP	MBP	HR	SBP	DBP	MBP	HR
Basal								
SHR	156 ± 3	132 ± 3	140 ± 3	297 ± 6	160 ± 4	137 ± 4	145 ± 4	325 ± 6
WKY	118 ± 3	90 ± 2	100 ± 1	362 ± 9	120 ± 3	92 ± 2	102 ± 2	373 ± 11
59 days after microinjection								
SHR LVV-hKir2.1	131 ± 5*	113 ± 4*	119 ± 4*	271 ± 2	133 ± 7*	114 ± 6*	120 ± 6*	320 ± 5*
SHR LVV-eGFP	172 ± 11	145 ± 11	154 ± 10	264 ± 5	177 ± 10	152 ± 11	160 ± 10	305 ± 7
WKY LVV-hKir2.1	117 ± 4	87 ± 3	97 ± 2	340 ± 10	121 ± 2	91 ± 4	101 ± 3	378 ± 12
WKY LVV-eGFP	114 ± 2	88 ± 3	96 ± 2	354 ± 5	116 ± 2	92 ± 3	100 ± 2	389 ± 2

Values are expressed as means ± SEM. Abbreviations: DBP, diastolic blood pressure; HR, heart rate; MBP, mean blood pressure; and SBP, systolic blood pressure; SHR LVV-hKir2.1, Spontaneously hypertensive rats microinjected with LVV-hKir2.1; SHR LVV-eGFP, Spontaneously hypertensive rats microinjected with LVV-eGFP; WKY LVV-hKir2.1, Wistar Kyoto rats microinjected with LVV-hKir2.1; WKY LVV-eGFP, Wistar Kyoto rats microinjected with LVV-eGFP. *$P < 0.01$, statistically significant difference between basal and day 59 values.

Table 2. Metabolic evaluation of spontaneously hypertensive rats before injection and 59 days afterwards

Group	ΔWeight (g)	Food (g)	Water (ml)	Faeces (g)	Urine (ml)
Before microinjection; basal conditions					
SHR LVV-eGFP	−1 ± 2.0	19 ± 3.5	27 ± 2.2	9 ± 2.1	11 ± 1
SHR LVV-hkir2.1	−1 ± 1.4	24 ± 1.3	40 ± 4.2	14 ± 3.1	16 ± 3.5
After microinjection (at 59 days)					
SHR LVV-eGFP	−3 ± 1.3	27 ± 1.6	31 ± 4.0	13 ± 1.5	12 ± 0.9
SHR LVV-hkir2.1	−1 ± 0.6	20 ± 0.6*	32 ± 4.9	8 ± 0.9	16 ± 2.1

Values are expressed as means ± SEM. Abbreviations: SHR LVV-hKir2.1, Spontaneously hypertensive rats microinjected with LVV-hKir2.1; SHR LVV-eGFP, Spontaneously hypertensive rats microinjected with LVV-eGFP; WKY LVV-hKir2.1, Wistar Kyoto rats microinjected with LVV-hKir2.1; WKY LVV-eGFP, Wistar Kyoto rats microinjected with LVV-eGFP. *$P < 0.05$, statistically significant difference between basal and day 59 values.

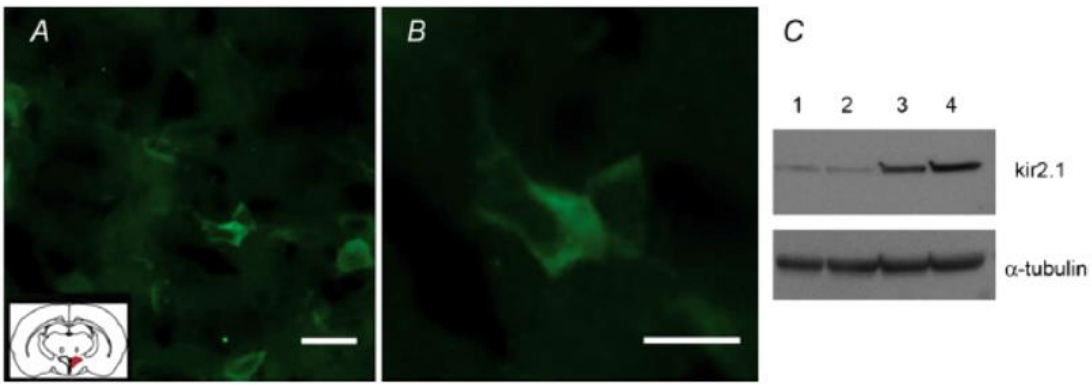

Figure 4. Lentiviral vector-mediated transduction of enhanced green fluorescent protein (eGFP) in the paraventricular nucleus
Confocal microscope images of eGFP-expressing cells in the paraventricular nucleus following injection of lentiviral vector into this site. Scale bar in *A* represents 20 μm and scale bar in *B* represents 10 μm. *C*, Western blot analysis of sham-treated SHRs (lanes 1 and 2) and LVV-hKir2.1 microinjected SHRs (lanes 3 and 4). Results show an overexpression of hKir2.1 in LVV-hKir2.1-microinjected SHRs. α-Tubulin was used as the housekeeping gene.

neurones, in order to evaluate its consequences upon long-term blood pressure regulation in an animal model of hypertension. We overexpressed a human inwardly rectifying potassium channel (hKir2.1) under the control of a synapsin promoter that was neurone specific (Duale *et al.* 2005*a*; Duale *et al.* 2005*b*). Lentivirus was used because its expression has been shown to be sustained within PVN neurones in the long term (Coleman *et al.* 2003). In previous studies, Duale *et al.* (2007) and Howorth *et al.* (2009) showed that hKir2.1 overexpression hyperpolarized the membrane potential of cultured cathecolaminergic PC12 cells by ~10 mV, which is expected to result in 'electrical silencing' of PVN neurones (Duale *et al.* 2007; Howorth *et al.* 2009). Similar overexpression strategies have been used to reveal that electrical silencing of neurones affected development *in ovo* (Yoon *et al.* 2008), neuronal activity *in vivo* (Okada & Matsuda, 2008) and the ability of neurones to make and maintain connections *in vivo* (Yu *et al.* 2004; Mizuno *et al.* 2007; Hendy, 2010). This virus-mediated approach has the advantage of being site specific and enabling overexpression in adulthood, which avoids the development of putative compensatory mechanisms associated with transgenic animals (Hendy, 2010).

Our results show that LVV-hKir2.1 treatment of the PVN in SHRs lowered SBP by ~15% (>20 mmHg). This decline of SBP, which was accompanied by a decrease in HR, was statistically confirmed at 30 days after the lentiviral microinjection and persisted until the animals were killed 60 days postinjection. Interestingly, the LF spectra of SBP (indicative of sympathoinhibition) occurred before the fall in SBP (i.e. 20 *versus* 30 days), suggesting a putative association between the changes in both variables. Furthermore, the fall in HF SBP is indicative of reduced respiratory modulation of arterial pressure and could include reduced respiratory–sympathetic coupling, a phenomenon known to raise total peripheral resistance in the SHR (Simms *et al.* 2009). In contrast, changes in diastolic BP were significant only after 50 days, suggesting the involvement of an additional mechanism. This reveals novel insight into the long-term control of arterial pressure in hypertension by the PVN. It also indicates that the system does not adapt. This could be explained by the associated improvement of baroreflex gain and/or a downregulation of peripheral chemoreflex responsiveness to stabilize lower levels of blood pressure, as we observed. We propose that these changes were a result of reduced electrical excitability of PVN premotor sympathetic neurones, but we cannot rule out reduced release of vasopressin and oxytocin. This is consistent with our neuroanatomical Western blot analysis confirming that hKir2.1 protein overexpression was within the PVN region. Interestingly, respiratory rate remained unchanged in all experimental groups, suggesting that there is no tonic excitatory drive from the PVN affecting this variable in hypertensive or normotensive rats. Additionally, we saw no tonic influence from the PVN on the resting arterial pressure level in normotensive rats, which contrasts with a previous acute *in vivo* study (Allen, 2002).

It is well accepted that neurogenic hypertension is accompanied by an impairment of the baroreceptor reflex (Grassi *et al.* 1998). Our data showed that depressing PVN neuronal activity improved baroreflex gain. Previous work from several authors has shown that during the course of an alerting reaction there is a decrease in baroreflex efficacy and a facilitation of the carotid chemoreceptor reflex due to modifications of synaptic integration at the level of the nucleus tractus solitarii; this might include mechanisms involving GABA and angiotensin II release within the nucleus tractus solitarii (Jordan *et al.* 1988; Spyer K, 1990; Silva-Carvalho *et al.* 1995*a*,*b*; Kasparov *et al.* 1998; Kasparov & Paton, 1999; Head & Mayorov, 2001; Rocha *et al.* 2003). Such an angiotensinogenic mechanism seems to be particularly active in pathophysiological conditions such as myocardial ischaemia and hypertension (Rocha *et al.* 2003; Rosário *et al.* 2003; Maximino *et al.* 2006), and its behaviour can be modulated by intervening pharmacologically on AT_1 receptors within the nucleus tractus solitarii (Kasparov *et al.* 1998; Kasparov & Paton, 1999; Rocha *et al.* 2003; Rosário *et al.* 2003). In fact, during myocardial ischaemia, AT_1 blockade reversed the remodelling of baroreceptor and chemoreceptor reflex function in a way similar to that elicited upon the overexpression of hKir2.1 in PVN neuronal cells (Rocha *et al.* 2003; Rosário *et al.* 2003).

The demonstration of a non-dipper blood pressure profile in animal models remains difficult, mainly due to the failure to establish a clear distinction between day and night values. This was confirmed in our study, because through PVN-induced sympathetic manipulations, we were only able to modify BP light–dark values of SHRs which approached those of WKY rats. However, we were unable to modify the day and night profile of BP value variations in both strains. This inability to define a light–dark profile in rats similar to the one set for human subjects may be due to the intermittent behaviour rats, with alternating awake and sleep periods in both the light and the dark phase. It is likely that the only way to define the light and dark phase profiles of rats better would be by monitoring of cerebral activity through EEG, which was outside the scope of the present work.

In conclusion, the present work shows that the intervention on central sympathoexcitatory neurone excitability through the genetic manipulation of expression of K^+ channels is able to alter peripheral blood pressure in the long term. This occurs by remodelling of the sympathetic outflow and restores the imbalance of peripheral reflex mechanisms that maintain cardiovascular homeostasis. Our data, from an

animal model, give insights into the pathophysiological mechanisms involved in the aetiology of neurogenic hypertension and provide novel hypothetical therapeutic interventions at both the central and the peripheral level of the autonomic nervous system to control sympatoexcitation.

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Additional Information

Competing interests

None declared.

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